REGULATORY MECHANISMS INVOLVED IN THE EXPRESSION OF BRAIN-DERIVED NEUROTROPHIC FACTOR AND GLIAL CELL LINE-DERIVED NEUROTROPHIC FACTOR

1996

BISHOP

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Abstract

Title of Dissertation: Regulatory mechanisms involved in the expression of brain-derived neurotrophic factor and glial cell line-derived neurotrophic factor

John F. Bishop, M.S., Doctor of Philosophy, 1996

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Brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) exert potent neurotrophic actions on several neuronal populations and are capable of protecting dopaminergic neurons in the brain from neurotoxin-induced degeneration. While many of the studies conducted to date have focused on the postsynaptic effects of BDNF and GDNF, very few have addressed the regulatory mechanisms involved in the expression of these factors.

In phase 1 of the project, five alternate first exons contained in the rat BDNF gene, including a novel one termed exon 1a, were isolated and found to be individually spliced to a common protein coding exon resulting in five separate transcripts differing at their 5′ ends. Subsequent experiments conducted in phase 2 indicated that expression of these five transcripts is differentially regulated in the brain regions investigated (substantia nigra, striatum, hippocampus and cerebellum), indicating differential utilization of alternate promoters. In addition, it was determined that long-term BDNF mRNA expression in the striatum was not altered by destruction of the presynaptic dopaminergic pathway and that calcium is a prominent second messenger involved in the regulation of BDNF and GDNF gene

transcription. Treatment of C6 glioma cells with A23187, forskolin + isobutyl-methylxanthine (IBMX) or 12-O-tetradecanoyl-phorbol-13-acetate (TPA) to elevate intracellular calcium, cAMP or protein kinase C activity, respectively, led to varying elevations in BDNF and GDNF transcript levels. A23187 treatment produced greater than 5-fold elevations in all of the mRNA species measured, which was largely attributed to an increased rate of transcription initiation. Forskolin + IBMX or TPA treatments lead to 2-fold and 3-fold increases in BDNF and GDNF mRNA levels, respectively. In phase 3, it was determined that the proximal 175 base pairs of the BDNF exon 1e promoter contains important positive regulatory elements and that the region positioned just upstream contains one or more silencers.

Understanding the mechanisms regulating BDNF and/or GDNF transcription should facilitate strategies to augment their production *in vivo* and allow the development of new approaches to the treatment of neurodegenerative disorders, such as Parkinson's disease.

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by

John F. Bishop

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DEDICATION

This dissertation is dedicated to my wife, Elizabeth, and to my family for their boundless love and support that have sustained me through the difficult times and allowed me to fully appreciate the impact of my work.

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ABBREVIATIONS

Abbreviation	Name
6-OHDA	6-hydroxydopamine
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
BDNF	brain-derived neurotrophic factor
β-gal	β-galactosidase
CaRE	calcium responsive element
CAT	chloramphenicol acetyltransferase
CRE	cyclic AMP responsive element
DHFR	dihydrofolate reductase
DMEM	Dulbecco's modified eagle's medium
DMSO	dimethysulfoxide
ELISA	enzyme-linked immunoassay
ERE	estrogen response element
GABA	γ-aminobutryic acid
GAPDH	glyceraldehyde phosphate dehydrogenase
GDNF	glial cell line-derived neurotrophic factor
GRE	glucocorticoid response element
MK-801	(+)-5-methyl-10,11-dihydro-5H-dibenzo-(a,d)-cyclohepten-5,10-imine-maleate
NBQX	2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxaline
L-Dopa	levodopa
LNGFR	low affinity nerve growth factor receptor
MPP^+	N-methyl-4-phenylpyridinium ion
MPTP	N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NMDA	N-methyl-D-aspartate
NGF	Nerve growth factor
NT-3	neurotrophin-3
NT-4	neurotrophin-4
NT-5	neurotrophin-5
ONPG	o-nitrophenyl-β-D galactopyranoside
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PKC	protein kinase C
RACE	Rapid Amplification of cDNA Ends
RPA	ribonuclease protection assay
RT	reverse transcription
SRE	serum responsive element
trk	tropomyosin receptor kinase
T_m	melting temperature
TPA	12-O-tetradecanoyl-phorbol-13-acetate

Chapter I Introduction

I-1. Overview of the neurotrophin family

I-1.a. General Characteristics of Nerve Growth Factor

Nerve growth factor (NGF), the prototypical neurotrophin, originally isolated by Levi-Montalcini and colleagues (Levi-Montalcini, 1987; see review in Henderson et al. 1993) has documented trophic actions on cholinergic neurons of the basal forebrain and many other neuronal systems (Hefti, 1986; Knusel et al. 1990; Knusel et al. 1991; Henderson et al. 1993). NGF isolated from mouse submaxillary gland, where it is found in very high concentrations, is comprised of three subunits, termed α-NGF, β-NGF and γ-NGF (Manness et al. 1994). In its high molecular weight form, termed the 7S complex, NGF is composed of dimers of the α and γ subunits associated with a single β subunit. Once β-NGF disassociates from the 7S complex in biological fluids, it forms a stable disulfide bonded dimer composed of identical 118 amino acid chains (Angeletti et al. 1971) that is solely responsible for biological activity (Varon et al. 1967).

I-1.b. Neurotrophic Actions of NGF

Inclusion of NGF in culture medium allows catecholaminergic neurons dissected out of sympathetic and primary sensory ganglia to survive significantly longer than neurons deprived of NGF, and this effect can be reversed by inclusion of NGF antibodies (Levi-Montalcini, 1987). In addition, these neurons exhibit enhanced differentiation, as evidenced by extensive neurite extension and increased

production of neurotransmitter biosynthetic enzymes (Thoenen and Barde, 1980). Whereas the primary targets of NGF in the peripheral nervous system are catecholaminergic neurons, this is not the case in the central nervous system. Administration of NGF to developing dopaminergic or adrenergic neurons in the midbrain was without effect on catecholamine synthesizing enzymes in these regions (Konkol et al. 1978). Thus, phenotypically similar populations of neurons are differentially responsive to NGF, depending on their physiological environment. The anatomically restricted response to NGF is primarily due to the restricted distribution of trkA NGF receptors (section I-1.e).

I-1.c. Retrograde Transport and Neuroprotective Actions of NGF

In studies evaluating the retrograde transport of NGF from hippocampus to basal forebrain nuclei, Schwab et al. (1979) demonstrated that this neurotrophin is taken up exclusively by cholinergic neurons. Subsequently, several additional cholinergic nuclei have been shown to transport NGF including nuclei located in the hippocampus, cortex, medial septum, diagonal band of Broca, and olfactory bulb, as well as interneurons in the striatum (Whittemore et al. 1986). NGF sensitive cholinergic neurons in the CNS respond to administration of exogenous NGF with an upregulation of choline acetyltransferase, the key enzyme involved in the production of acetylcholine (see Maness et al. 1994 for review). Subsequently, it was found that administration of NGF to rats prior to septohippocampal lesions protects cholinergic cell bodies located in the septum from degeneration (Morse et al. 1993). When NGF was injected intracerebroventricularly, 68% of the cholinergic cell bodies in the septum survived fimbria transection, as opposed to 28% survival after injection of vehicle. Direct injection of NGF into the septum was even more

efficacious, allowing 86% of the septohippocampal neurons to survive. In culture, these neurons survive approximately twice as long in the presence of NGF as opposed to vehicle and exhibit signs of enhanced differentiation (Alderson et al. 1990). Thus, NGF is an important target-derived factor for maintenance of cholinergic neurons in the CNS, which makes it a candidate for treatment of neurodegenerative disorders involving cholinergic nuclei, such as Alzheimer's disease.

I-1.d. Other Neurotrophins

Following the reports on the trophic actions of NGF and the development of NGF antibodies, several investigators reported that conditioned medium from neuronal and glial cell cultures contained unidentified growth factors that were not affected by inclusion of NGF antibodies (Barde et al. 1978, 1980). These findings led to the isolation and cloning of additional growth factors homologous to NGF, including brain-derived neurotrophic factor (BDNF, Barde et al. 1982; Leibrock et al. 1989), neurotrophin-3 (NT-3, Maisonpierre et al. 1990), neurotrophin-4 (NT-4, Hallbrook et al. 1991) and its mammalian homolog, neurotrophin-5 (NT-5, Berkemeier et al. 1991). These factors are highly conserved across animal species and several structural characteristics, such as the presence of six cysteine residues involved in 3 conserved disulfide bonds, are found in all members of the neurotrophin family (Ebendal, 1992). Each of these factors has distinct, as well as overlapping trophic and/or neuroprotective actions (Hyman et al. 1994; Koliatsos et al. 1993; Knusel et al. 1991; Ip et al. 1993). For example, although NGF is an important trophic factor for cholinergic neurons of the basal forebrain and sensory neurons in the dorsal root ganglion, BDNF has similar effects on both of these

neuronal populations and has potent trophic and protective actions on several other neuronal phenotypes as well (Barde et al. 1982; Hyman et al. 1991; Knusel et al. 1991). Thus, BDNF supports the survival and differentiation of a wider subset of neurons than those supported by NGF, making it an attractive target for development of novel approaches to the treatment of neurodegenerative disorders. As discussed in detail in section I-6, BDNF is an especially likely candidate for treatment of Parkinson's disease, owing to its potent trophic and neuroprotective effects on dopaminergic neurons implicated in this disorder. Neurotrophic factors are thought to function primarily as classic target-derived growth factors, which are secreted from "target" cells, bind to receptor complexes on neurotrophin-requiring cells and transported to their perikarya in retrograde fashion.

I-1.e. Neurotrophin Receptors

The neurotrophin receptors include the low affinity nerve growth factor receptor (LNGFR, Chao et al. 1986; Radeke et al. 1987) and members of the recently described tropomyosin receptor kinase (*trk*) proto-oncogene family that have important roles in signal transduction in addition to their roles in retrograde transport of neurotrophins. Prior to the isolation and cloning of the *trk* receptor family, studies of neurotrophin receptors were focused on a 75-80 kDa intrinsic membrane protein (p75), termed the LNGFR. This protein is expressed by numerous neurons and is capable of binding all of the neurotrophins described to date, albeit with low affinity (K_d approximately 10^{-9} M, Bothwell, 1991). The cytoplasmic domain of this protein is small, as compared to other receptors, and transfection studies have indicated that it has weak intrinsic trophic functions (see

Bothwell, 1991 for review). However, incubation of NGF-responsive cells with antibodies previously shown to block NGF binding to p75 have no effect on the trophic actions of NGF (Weskamp and Reichardt, 1991). This work suggested the existence of another NGF receptor, and in 1989, Martin-Zanca et al. (1989) reported that the *trk* proto-oncogene (*trk*A) encoded a 140 kDa tyrosine kinase that was shown to bind NGF. Subsequent studies have shown that trkA is required for high affinity NGF binding (K_d approximately 10^{-11} M) and is primarily responsible for the biological actions of NGF (Cordon-Cardo et al. 1991; Ibanez et al. 1992). However, other investigators have suggested that high affinity NGF binding requires a complex of p75 and trkA (see Bothwell, 1991 for review).

Since the neurotrophins are structurally similar (Ebendal, 1992), it was thought that the high affinity receptors for BDNF, NT-3 and NT-4/5 would resemble trkA. In 1991, investigators from two different laboratories reported the isolation and cloning of *trk*B, which is the primary receptor for BDNF and NT-4/5 and can also bind NT-3 (Squinto et al. 1991; Soppet et al. 1991). Later that year, another *trk*-like molecule was cloned, termed *trk*C (Lamballe et al. 1991) and subsequently shown to be the primary high affinity receptor for NT-3 (Klein et al. 1992).

After a neurotrophin binds to the ligand recognition domain, trk receptors form homodimers leading to autophosphorylation and activation of cytoplasmic tyrosine kinase domains in a process involving interaction with the LNGFR (Bothwell, 1991; Eide et al. 1993). Activation of the intrinsic tyrosine kinase leads to mitogenic and/or differentiative signalling cascades in receptor-bearing cells (Ip and Yancopoulos, 1994). Thus, release of NGF from granule cells residing in the hippocampus activates trkA receptors in the terminals of the cholinergic septohippocampal neurons. Ensuing signal transduction mechanisms, involving

protein phosphorylation, could then protect these neurons from potential pathology, as well as encourage deployment of adaptive functions, such as synaptic reorganization or sprouting. trk receptors are expressed by numerous neuronal phenotypes and the distribution of the trk subtypes corresponds well with the distribution of neurotrophin sensitive neurons. For example, large and medium-sized cholinergic neurons in the septohippocampal formation express trkA receptors and respond to NGF (Merlio et al. 1992). Similarly, dopaminergic neurons in the substantia nigra, known to respond to BDNF (see section I-3.a), express trkB receptors (Merlio et al. 1992).

I-2. Brain-Derived Neurotrophic Factor

I-2.a.General Characteristics of Brain-Derived Neurotrophic Factor

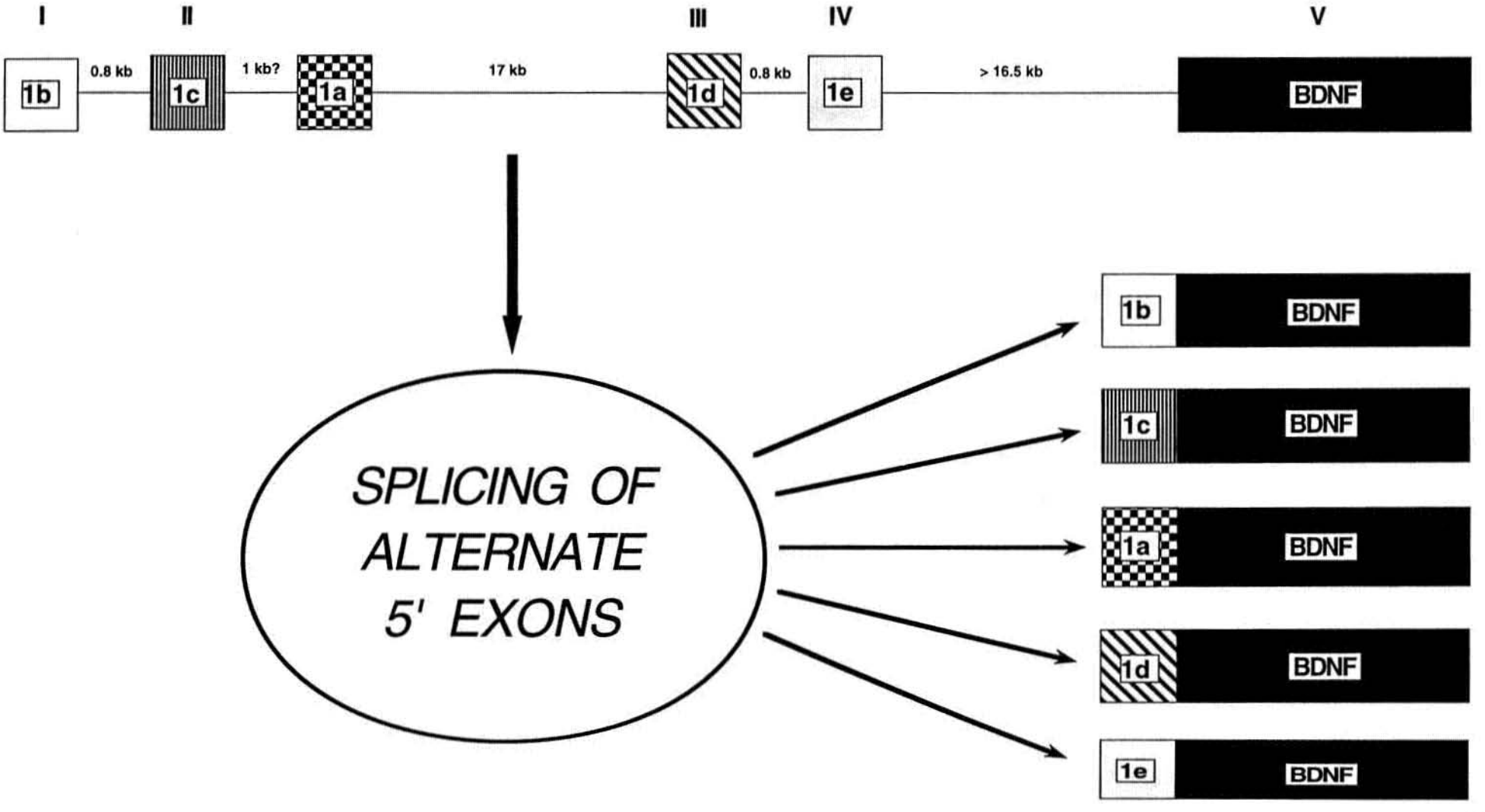
Brain-Derived Neurotrophic Factor (BDNF) is a 119 amino acid basic protein derived from a 252 amino acid precursor (Hohn et al. 1990). It is structurally similar to NGF and the other members of the neurotrophin family, exhibiting at least 50% homology with each of them. Other similarities include the conservation of six cysteines positioned to allow the formation of three crucial disulfide bridges and the fact that all neurotrophins form stable dimers in solution (Gotz et al. 1992; Radziejewski et al. 1992). BDNF is the most conserved member of the neurotrophin family across species and is identical in mouse, rat, pig and man (Maisonpierre et al. 1991). Mature BDNF is secreted from a variety of cells in contact with target neurons, including, in some instances, the target neuron itself (Lindsay et al. 1994). It then binds to trkB receptors expressed by these neurons leading to physiological actions similar to those described for activation of trkA receptors. An added

complexity in this process stems from the existence of truncated versions of the trkB receptor that lack the intrinsic kinase domain (Squinto et al. 1991; Yan et al. 1994). It is thought that intracellular signals generated from these truncated receptors are weak at best and that they probably function either to prevent access of BDNF to certain extracellular compartments, to concentrate BDNF in these compartments for later utilization or perhaps as mediators of cell-cell interactions (Eide et al. 1993). It is interesting to note that, while full length trkB is found primarily in neurons, the truncated forms of this protein appear to be expressed mainly in cells of the choroid plexus, in ventricular ependymal cells and in glial cells (Yan et al. 1994). Thus, access of BDNF to neurons may, to some extent, be under the control of non-neuronal cells determined by which trkB splice variants are expressed.

I-2.b. The Molecular Biology of BDNF

The BDNF gene consists of multiple noncoding first exons that are alternatively spliced with a common 3′ protein coding exon (Ohara et al. 1992; Timmusk et al. 1993; Nakayama et al. 1994). Four separate 5′ exons termed exons I,II,III and IV were localized in the rat BDNF gene, each of which is transcribed from a different promoter (Timmusk et al. 1993; Nakayama et al. 1994; see Figure 1). The schematic shown in Figure 1 uses both the exon naming convention of Timmusk et al. (1993) and a novel one developed in the present dissertation prior to the publication of the report by Timmusk and coworkers. In all, eight BDNF transcripts have been identified resulting from combinations of the four alternate first exons and usage of two different poly(A)$^+$ signal sequences (Timmusk et al. 1993). Use of the downstream poly(A)$^+$ signal leads to inclusion of an additional 2.6 kb of 3′

Figure 1. Schematic representation of the splicing of rat BDNF alternate first exons to the BDNF coding exon. Based on published information and the present work, alternative splicing mechanisms generate at least five transcripts, each containing a different 5′ sequence. The exon 1a sequence discovered in the present work is positioned between exons 1c and 1d on the basis of homology with unpublished genomic sequence (T. Timmusk, personal communication). Both the exon labeling conventions selected in this dissertation (alternate exons 1a-1e) and by Timmusk and colleagues, who used roman numerals (Timmusk et al. 1993), are shown.


I
II
III
IV
V
1b
0.8 kb
1c
1 kb?
1a
17 kb
1d
0.8 kb
1e
> 16.5 kb
BDNF
SPLICING OF
ALTERNATE
5' EXONS
1b
BDNF
1c
BDNF
1a
BDNF
1d
BDNF
1e
BDNF

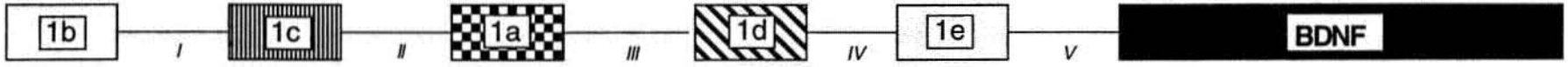
noncoding sequence in BDNF transcripts. Thus, activation of the exon 1a promoter, for example, would allow transcription of exon 1a and all genomic sequences between this exon and the end of the BDNF 3′ noncoding region. This very long transcript is then edited, removing exons 1d and 1e, as well as intronic sequences and 3′ noncoding sequences, via a process depicted in Figure 2. In this case, the mature mRNA contains exon 1a directly spliced to the protein coding exon. Transcription arising from the other promoters presumably occurs in a similar manner, and produces the alternate transcripts depicted in Figure 1. The existence of multiple BDNF promoters raises the possibility of differential regulation of transcription, producing different BDNF transcript types depending on the signal transduction system(s) activated in a particular cell. Conversely, different cell types may maintain the BDNF gene in different functional states, allowing only certain BDNF promoters to become active.

I-2.c Localization of BDNF mRNA Expression

In the adult rat brain, BDNF mRNA is found in high concentrations, relative to other neurotrophins, in the hippocampus, cerebral cortex, cerebellum, midbrain and diencephalon (Maisonpiere et al. 1990). In the same study, it was revealed that BDNF mRNA levels in the periphery are moderately enriched in heart, lung and muscle tissues. Studies meant to determine the cell types expressing BDNF in the brain have indicated that a major source in the midbrain is the nigrostriatal dopaminergic neurons, themselves (Seroogy et al. 1994). These studies employed double labeling immunohistochemical techniques to identify tyrosine hydroxylase positive neurons that also express BDNF mRNA. The results confirmed prior speculations that the dopaminergic neurons might produce BDNF (Ceccatelli et al.

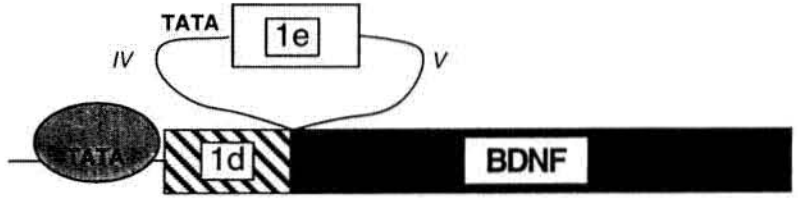
Figure 2. Schematic representation of the process by which individual rat BDNF alternate exons are spliced to the coding exon. Activation of one of the alternate promoters, which is indicated by the protein-bound TATA element in the figure, leads to production of a primary mRNA molecule containing all of the exons (and introns) between the active promoter and 3′ non-coding exons (not shown). Prior to production of the final splice variant, these exon and intron regions are apparently looped out and excised allowing ligation of the correct exon to the coding exon. The mechanism responsible for ensuring that only the exon adjacent to the activated promoter is included in the mature mRNA, in spite of the fact that individual cells can produce more than one alternate BDNF transcript is unknown.

Hypothetical Splicing of Alternate 5' Exons in the Rat BDNF Gene

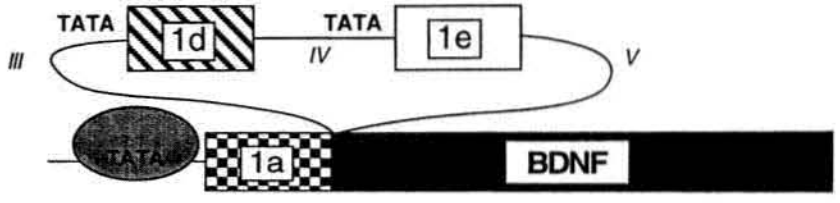


When the **Exon 1e Promoter** is Activated...

When the **Exon 1d Promoter** is Activated...



When the **Exon 1a Promoter** is Activated...



1991) and indicated that between 10% and 50% of these neurons in the subtantia nigra and ventral tegmental area produce BDNF. The recent finding that these neurons also express trkB (Merlio et al. 1992) suggests that a paracrine or autocrine mechanism is involved in the maintenance of the dopaminergic phenotype and/or survival of these cells and raises the possiblity that degeneration of nigrostriatal neurons seen in Parkinson's disease is a result of decreased expression of BDNF in these neurons.

I-2.d. Evidence for Paracrine or Autocrine Regulation of Neuron Survival by BDNF

It is interesting to note that cultured sensory neurons isolated from the dorsal root ganglion, which also produce BDNF, require this neurotrophin for survival (Acheson et al. 1995). These investigators down-regulated BDNF expression in low density neuronal cultures by incubation with BDNF antisense oligonucleotides and compared the rate of cell death to that in cultures incubated with control oligonucleotides. They found that neurons exposed to the BDNF-specific oligonucleotides died significantly faster than neurons exposed to control oligonucleotides, suggesting that continuous release of BDNF is required for survival. In a similar approach, Ghosh et al. (1994) generated antibodies specific to BDNF and showed that these antibodies can reverse calcium-dependent survival of embryonic cortical neurons in culture. Since the expression of BDNF in these neurons was also calcium-dependent, they suggested that BDNF may function as an autocrine or paracrine factor during cortical development. In combination, the studies outlined above provide strong evidence for a physiological role for BDNF in the survival of different neuronal populations and evidence for the existence of

paracrine or autocrine mechanisms.

I-2.e. Localization of Alternate BDNF Transcripts

In addition to studies of the brain regions and cell types expressing BDNF, recent studies have examined the production and distribution of alternate BDNF transcripts in various rat tissues and brain regions and have suggested that these transcripts are expressed in a tissue-selective manner (Timmusk et al. 1993; Metsis et al. 1993; Nakayama et al. 1994; Kokaia et al. 1994; Lindvall et al. 1994). Timmusk and coworkers (Timmusk et al. 1993) reported that rat brain regions, including the cerebral cortex and several subregions of the hippocampus, express BDNF transcripts containing exons I,II and III, but are devoid of exon IV. They also reported that BDNF expression in heart, lung and other peripheral tissues is primarily due to exon IV-containing transcripts. In a similar study, Nakayama and colleagues (Nakayama et al. 1994) also reported that alternate BDNF transcripts are initiated from multiple alternate promoters in tissue-selective manner. Transfection studies conducted by Timmusk and coworkers provided further evidence for tissue-selective transcription via differential activation of alternate BDNF promoters. They transfected chloramphenicol acetyltransferase (CAT) reporter constructs containing 5′ flanking regions adjacent to each of the original four BDNF exons (I-IV) into neuronal and non-neuronal cell lines. As suggested by their studies of endogenous expression of these exons, the results indicated that promoters I-III were primarily active in cells of neural origin, while the exon IV promoter was only active in cells derived from peripheral (non-neuronal) tissues (Timmusk et al. 1993). In subsequent studies of the response of the BDNF gene to various pathological conditions (see section I-4), further evidence for cell type and insult-specific

activation of alternate BDNF promoters was presented.

I-3 The Physiological Roles of BDNF

I-3.a. Actions of BDNF on Dopaminergic Neurons

Infusion of BDNF into the rat brain produces significant behavioral, biochemical and electrophysiological changes in dopaminergic systems that are not seen after injection of vehicle or NGF (Altar et al. 1992; Sauer et al. 1994; Shen et al. 1994; Shults et al. 1994). Altar et al. (1992) have shown that unilateral supranigral injections of BDNF lead to increased dopamine turnover in the ipsilateral striatum and to amphetamine-induced contralateral rotations, suggesting that dopaminergic transmission is enhanced on the injected side on the brain. This hypothesis is supported by the results of Shen et al. (1994), which indicated that identical BDNF injections lead to significant (65-98%) increases in the number of spontaneously active dopaminergic neurons in the nigra and increased the average firing rate by 32%. In addition, a single unilateral injection of BDNF into the medial substantia nigra, pars compacta led to significant increases in amphetamine-induced rotations that persisted for up to 12 months (Shults et al. 1994), indicating that the effects of BDNF are long-lived. Furthermore, BDNF administered into the lateral ventricles or injected directly into fetal mesencephalic cells transplanted into the the striata of 6-hydroxydopamine (6-OHDA) lesioned rats essentially abolishes amphetamine-induced rotations (Sauer et al. 1993). 6-OHDA is a neurotoxic dopamine analog that is taken up by nigral dopaminergic cells where it is metabolized to highly reactive oxygen free radicals that cause cell death. Thus, BDNF can enhance the function of transplanted dopaminergic cells and may be useful as an adjunct treatment in

clinical transplantation procedures. Because a single oral dose of levodopa reportedly increases striatal BDNF expression (Okazawa et al. 1992) it is possible that target cells of the nigrostriatal tract are responsive to alterations in presynaptic dopamine release. *In toto*, these findings raise the intriguing possibility that increasing BDNF concentrations in the substantia nigra or striatum may have beneficial effects on nigrostriatal dopamine neurons with potential therapeutic applications in the treatment of Parkinson's disease.

I-3.b. Induction of BDNF mRNA Expression by Environmental Stimuli

Other evidence for a physiological role of BDNF in the CNS is provided by reports that environmental changes which alter neuronal physiology lead to alterations in the expression of BDNF mRNA in neurons (Castren et al. 1992; Falkenberg et al. 1992; Neeper et al. 1995). When rats were deprived of light for seven days, BDNF mRNA levels in neurons of the visual cortex were decreased approximately 50%, whereas NGF mRNA levels were unchanged (Castren et al. 1992). When light deprived rats were returned to a normal diurnal cycle, the BDNF mRNA expression returned to normal within one hour after exposure to light. In a similar paradigm, Falkenberg et al. (1992) subjected rats to either an enriched or impoverished environment and demonstrated significant positive effects of the enriched environment on behavioral measures such as locomotion and the acquisition of spatial memory. They also reported that BDNF mRNA levels in the hippocampi of enriched rats that were sacrificed just after spatial memory testing were significantly higher than levels measured in non-tested enriched rats. This latter result is congruent with the results of Castren et al. (1993) and others (Patterson et al. 1992), who showed that induction of long-term potentiation in rats

invivo leads to significant increases in BDNF expression in dentate granule cells of the hippocampus. Long-term potentiation has been implicated in the consolidation and storage of memory (Bliss and Collingridge, 1993), and since long-term potentiation is impaired in knockout mice lacking BDNF (Korte et al. 1995), BDNF might have a functional role in this process. Interestingly, it has recently been shown that infusion of BDNF can enhance synaptic function both in the hippocampus (Levine et al. 1995; Kang and Schuman, 1995) and at the neuromuscular junction (Lohof et al. 1993), and BDNF mRNA levels are reduced in the hippocampi of persons afflicted with Alzheimer's disease (Phillips et al. 1991), suggesting again that BDNF may mediate learning and memory processes. In combination, the aforementioned studies suggest that BDNF is involved in the neural response to changes in environmental stimuli and further suggest that these neurotrophic effects are exerted on cholinergic systems.

As additional testimony to the link between environmental stimuli and BDNF mRNA expression, Neeper et al. (1995) reported that physically active rats have increased hippocampal BDNF mRNA levels, as compared to more lethargic rats. Since infusion of BDNF (section I-6.a) has documented trophic effects on both cortical and hippocampal neurons, it is possible that upregulation of BDNF transcripts in these regions by sensory stimulation facilitates the function of target neurons. Moreover, a recent report that BDNF is expressed in the sensory epithelium of the developing organ of Corti of the rat (Wheeler et al. 1994) and is required for normal development of vestibular systems (Ernfors et al. 1994; Jones et al. 1994) in mice lends further credence to the notion that BDNF plays a role in the response of CNS circuits to external stimuli.

I-3.c. Neurotrophic actions of BDNF on GABAergic and Serotonergic Neurons

To determine if BDNF has trophic actions on neuronal phenotypes other than cholinergic or dopaminergic, several investigators have studied the effects of BDNF on GABAergic and serotonergic neurons in mesencephalic and striatal cultures (Hyman et al. 1994; Martin-Iverson et al. 1994; Ventimiglia et al. 1995). Hyman et al. (1994) reported increased GABA uptake and expression of GAD in fetal mesencephalic cultures after exposure to BDNF. Similarly, Martin-Iverson et al. (1994) reported that continuous unilateral infusion of BDNF into the substantia nigra led to significant increases in dopamine turnover and amphetamine-induced behaviors, such as contralateral rotations, as well as a previously unreported increase in striatal serotonin turnover. More recently, Ventimiglia et al. (1995) found that treatment of embryonic striatal cultures with BDNF results in enhanced differentiation and/or survival of GABAergic neurons. Thus, in addition to the well-documented effects on dopaminergic and cholinergic neurons, BDNF appears to have important trophic effects on GABAergic and serotonergic neurons as well.

I-3.d. Effects of BDNF on Neuropeptide mRNA Expression

Other studies concerning the physiological role of BDNF in the brain have focused on the effects of exongenous BDNF on the biosynthesis of neuropeptide transmitters in target neurons. Sauer and coworkers (Sauer et al. 1994) injected rat striata with BDNF daily for one week (12 ug per day) and measured striatal neuropeptide expression. They found that both preproenkephalin and preprotachykinin mRNA expression was significantly elevated by BDNF treatment. Similarly, Nawa et al. (1993) measured the levels of neuropeptide Y, somatostatin,

cholecystokinin and GABA in cultured cortical neurons after exposure to BDNF (20 ng/ml) for five days. This treatment produced significant elevations in all of these neurotransmitters, whereas other neurotrophins, such as NT-3 and NGF, had little (NT-3) or no (NGF) effect. Croll et al. (1994) obtained results similar to those of Nawa and coworkers after infusion of BDNF into the hippocampus, cortex or striatum of adult rats. Levels of all of the peptides measured, including neuropeptide Y, cholecystokinin, substance P and somatostatin, were upregulated in all brain regions assayed, with the exception of dynorphin in the hippocampus, which was down-regulated. Thus, BDNF appears to be involved in the regulation of neurotransmitter levels in target neurons and may therefore play a role in transsynaptic signal transduction.

I-4 BDNF and Brain Injury

I-4.a. Effects of Lesion-Induced Seizures and Kindling on the Expression of NGF and BDNF in the Hippocampus

It has been known for some time that seizure activity in the brain results in dramatic elevations in neurotrophin gene expression (Gall and Isackson, 1989; Isackson et al. 1991; Ernfors et al. 1991), via a mechanism involving excitatory amino acid pathways (Lindvall et al. 1994). Gall and coworkers demonstrated that induction of hippocampal seizures by electroyltic lesion of the dentate gyrus hilus results in a 20- to 28-fold increase in NGF mRNA expression in adjacent areas of the granule cell layer 2.5-6 hours after seizure onset (Gall and Isackson, 1989). Using the same paradigm, these investigators found that BDNF mRNA expression was increased more than 9-fold in the dentate gyrus in the same time frame and was also

dramatically increased in areas CA1 and CA3, as well as regions of the cerebral cortex (Isackson et al. 1991). Shortly thereafter, Ernfors et al. (1991) reported similarly rapid and large increases in BDNF mRNA expression in the same areas of the hippocampus and cerebral cortex after repeated subconvulsive electrical stimulations of the ventral hippocampus (kindling). In addition to seizures evoked by lesions or kindling, pentylenetetrazol-induced epileptic seizures also lead to rapid increases in BDNF transcripts, primarily in the dentate gyrus, amygdala and piriform cortex (Humpel et al. 1993). These investigators also reported that BDNF-like immunoreactivity was significantly decreased in the dentate gyrus and hilus 3 hours after seizure onset, suggesting that BDNF is released from neurons in these regions during seizures. Surprisingly, BDNF mRNA levels are also elevated by decreased electrical activity. Kokaia and coworkers (Kokaia et al. 1993) found that single or repeated application of KCl to the surface of the rat cerebral cortex, which induces spreading depression of electrical activity, produces significant increases in BDNF mRNA expression in superficial layers of the cortex. Since this effect was significantly attenuated by prior treatment with an N-methyl-D-aspartate (NMDA) receptor antagonist, glutamatergic mechanisms appeared to be involved.

I-4.b. Effects of Glutamate Receptor Agonists on BDNF mRNA Expression

Several investigators have reported that injection of excitotoxic compounds, such as the non-NMDA glutamate receptor agonist, kainic acid, induces behavioral seizures and large elevations in BDNF mRNA expression in certain populations of cortical and hippocampal neurons (Ballarin et al. 1991; Lindefors et al. 1992; Dugich-Djordjevic et al. 1992). Kainic acid binds to ionotropic non-NMDA receptors and stimulates glutamate release from target neurons, which then has transient

excitatory actions on neighboring neurons (Sperk, 1994). Within thirty minutes after the onset of kainic acid-induced seizures in rats, large increases in BDNF mRNA levels occur in several regions of the hippocampus and cerebral cortex (Dugich-Djordjevic et al. 1992). Peak increases seen after 4 hours ranged from 2.4 to 9-fold, with the larger increases seen in hippocampal subregions (dentate gyrus and areas CA1 and CA3), with smaller increases in the cerebral and piriform cortices. In a similar study, Ballarin et al. (1991) reported a 40-fold increase in BDNF mRNA expression in the hippocampus seven hours after a direct injection of kainic acid into the dorsal portion of this region.

As mentioned previously, BDNF transcripts that are expressed in hippocampal subregions may be derived from multiple promoters and therefore may be differentially expressed. In their investigations of the transcriptional mechanisms involved in excitotoxin-induced increases in BDNF mRNA levels, several investigators examined the contribution of each of the alternate transcripts to the observed increases (Timmusk et al. 1993; Metsis et al. 1993; Nakayama et al. 1994). Following kainic acid treatment, transcripts containing exons I,II and III are elevated in the dentate gyrus, whereas only exon III is elevated in CA1 and both I and III are elevated in CA3. Since these effects were differentially sensitive to glutamatergic and GABAergic receptor blockade (Metsis et al. 1993, see section I-5.b), it appears that determination of which BDNF promoter that is ultimately activated after kainic acid depends on both the specific neuronal population and the glutamate receptor subtypes expressed thereby.

Lindefors et al. (1991) compared the effects of intrahippocampal injections of kainic acid to the effects of quisqualate injected into the entorhinal cortex on BDNF mRNA expression. Both of these treatments produced large increases in BDNF transcripts in subregions of the hippocampus, but the pattern of expression varied.

Stimulation of hippocampal afferents with quisqualate led to increases primarily in area CA1, while the largest increases seen after injection of kainic acid were found in the dentate gyrus. Since the increases induced by quisqualate were prevented by pretreatment with the muscarinic antagonist, scopolamine, the investigators suggested that the afferent pathway contains a cholinergic component. In a more recent study, Rocamora et al. (1994) found that intrahippocampal injection of the endogenous excitotoxin, quinolinic acid, which induces seizures and local neurodegeneration, leads to dramatic increases in BDNF mRNA expression in the dentate gyrus and other regions contralateral to the injection site. This effect, which occurred between 3 and 5 hours post injection, was attenuated by pretreatment with the NMDA antagonist, (+)-5-methyl-10,11-dihydro-5H-dibenzo-(a,d)-cyclohepten-5,10-imine-maleate (MK-801), suggesting that trans-synaptic glutamatergic pathways were involved in the observed effects.

I-4.c. Effects of Ischemia and Hypoglycemic Coma on BDNF mRNA Expression

In other studies it was found that BDNF mRNA expression in the hippocampus and other brain regions is elevated after other types of brain insults, including ischemia and hypoglycemia (see Lindvall et al. 1994 for review). Lindvall et al. (1992) reported that subjection of rats to cerebral ischemia increased BDNF and NGF mRNA levels in the dentate gyrus at 2 and 4 hours after 2 minutes of bilateral carotid artery occlusion, respectively. As reported by others after treatment with excitotoxic compounds (Rocamora et al. 1994) or electrolytic lesions (Gall, 1993), NT-3 mRNA levels were significantly decreased at this time suggesting, once again, that neurotrophic factors are differentially regulated in the hippocampus (Lindvall et al. 1994). When 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxaline (NBQX), a non

NMDA glutamate antagonist, was administered prior to ischemia, a small significant decrease in BDNF mRNA expression resulted and since MK-801 was ineffectual, the investigators suggested that post-ischemic BDNF transcriptional mechanisms involve non-NMDA glutamate receptors (Lindvall et al. 1992). Kokaia et al. (1994) confirmed the results of Lindvall and coworkers and also reported that the observed increases in BDNF mRNA levels in the dentate gyrus was due primarily to activation of the BDNF exon III promoter.

The results of other experiments reported by Lindvall and colleages (Lindvall et al. 1992; Kokaia et al. 1994) indicated that increased BDNF mRNA expression occurring after hypoglycemic coma or kindling-induced seizures was also attributed to differential activation of alternate BDNF promoters. Insulin-induced hypoglycemic comas of 1 or 30 minutes duration led to 2 to 3-fold increases in BDNF transcripts 2 hours after the insult that was confined to the dentate gyrus (Lindvall et al. 1992). Subsequent experiments suggested that the increase was primarily due to activation of the exon III promoter, with a minor contribution by the exon I promoter (Kokaia et al. 1994). Kindling-induced seizures led to increased BDNF mRNAs in the dentate gyrus that were attributed to activation of exon I,II and III promoters, while in other areas of the hippocampus (CA3) and cortex (amygdala, piriform cortex and neocortex), transcripts arising from the exon I and III promoters were observed. In the CA1 region of the hippocampus, only exon III-containing transcripts were seen.

In combination, studies of the effects of neuropathological insults on BDNF mRNA expression in hippocampal and cortical neurons suggest that these transcripts are regulated in a region and insult-specific manner. The fact that neurons residing in the dentate gyrus are particularly resistant to ischemic and epileptic degeneration (Siesjo et al. 1988; Auer and Siesjo, 1988), may be due to the

rapid increases in exon I,II and III-containing BDNF transcripts seen in this region in response to these insults. Kokaia et al. (1994) noted that the duration of seizure activity required to produce neuronal necrosis in the hippocampus is longer than that required by other insults, which might be due to the more rapid accumulation of BDNF mRNAs seen after this insult. Rapid accumulation of BDNF mRNA would conceivably lead to rapid accumulation of BDNF protein, which has important neuroprotective actions on neurons (sections I-6.a, I-6.b). Accordingly, slower accumulation of BDNF in other regions of the hippocampus might provide insufficient protection, leading to neuronal death.

I-5 Receptors and Signal Transduction Pathways Involved in the Regulation of BDNF mRNA Expression

I-5.a. Effects of Neurotransmitter Receptor Ligands and Second Messenger-Inducing Compounds on BDNF mRNA Expression

In a series of reports, Zafra et al. (1990, 1991, 1992) studied the effects of numerous neurotransmitter receptor agonists and antagonists on BDNF and NGF expression in hippocampal neurons, both in culture and *invivo*. Whereas most of the compounds investigated were without effect, including nicotine, serotonin, dopamine, norepinephrine, substance P and somatostatin, BDNF mRNA was elevated more than 2-fold by the muscarinic agonist, carbachol, and more than 10-fold by KCl or kainic acid (Zafra et al. 1990). The effect of carbachol was prevented by cotreatment with atropine. The kainic acid-induced increase in BDNF transcripts was abolished by the calcium channel blocker, nifedipine and by the calmodulin antagonist, W7, implicating cholinergic and calcium-mediated mechanisms,

respectively (Zafra et al. 1992). Further investigations indicated that, while calcium mobilization is the primary signal transduction event coupling excitation to BDNF mRNA expression in hippocampal neurons, smaller elevations of these transcripts induced by the protein kinase C activator, TPA, and synergistic effects of the adenylate cyclase activator, forskolin, in combination with kainic acid were also reported. These investigators also reported significant increases in BDNF mRNA levels in cultures of neonatal astrocytes after treatment with the calcium ionophore, ionomycin, or with TPA (Zafra et al. 1992), and synergistic effects of cotreatment with forskolin and ionomycin were also observed.

I-5.b. Opposition of the Glutamatergic and GABAergic Systems in the Regulation of BDNF mRNA Expression

In studies conducted *invivo*, Zafra and colleagues (1991) found that rat BDNF mRNA levels in hippocampal neurons are regulated by the interactions of excitatory glutamatergic signals and inhibitory GABAergic signals. Just as excitation of these neurons with glutamate agonists or blockade of these actions with glutamate antagonists leads to increased or decreased BDNF mRNA expression, respectively, changes in GABAergic inhibitory tone also affects the expression of these transcripts. Activation of GABAergic receptors by systemic injections of muscimol or diazepam reduced BDNF mRNA levels in the hippocampus, while injections of bicuculline, a GABA receptor blocker, led to large elevations in BDNF transcripts (Zafra et al. 1991). Thus, perturbation of the balance between excitatory and inhibitory transmitter systems in the hippocampus results in alterations in BDNF mRNA expression.

To determine the roles of alternate BDNF promoters involved in the

maintenance of this balance, Metsis and coworkers (1993) examined the effects of pretreatment of rats with the glutamate receptor antagonists NMDA or α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) on kainic acid-induced BDNF transcripts arising from each of the four BDNF promoters. The results showed that promoters I,II and III were activated via stimulation of both NMDA and non-NMDA glutamate receptors in the neocortex, piriform cortex and amygdala whereas only non-NMDA receptors were involved in activation of these promoters in the *hippo*campus. Treatment with bicuculline increased the expression of exons I and III in the dentate gyrus and the muscarinic receptor agonist pilocarpine increased exon I expression in the neocortex. The authors concluded that alternate BDNF promoters are differentially regulated by the activities of glutamate and GABA receptor subtypes and that this regulation varies across brain regions (Metsis et al. 1993).

I-6 Neuroprotective Actions of BDNF

I-6.a. Neuroprotective Actions of BDNF on Cholinergic Neurons

Stemming from reports that BDNF mRNA levels are dramatically increased under neuropathological conditions, a large number of studies have been conducted to determine if BDNF has neuroprotective actions on axotomized neurons and neurons exposed to neurotoxins (Knusel et al. 1992; Oppenheim et al. 1992; Sendtner et al. 1992; Yan et al. 1992; Morse et al. 1993; Chiu et al. 1994; Lindsay et al. 1994). Transection of septohippocampal neurons leads to a 72% loss of cholingergic cell bodies that is reduced to 40% and 14% by concurrent intraseptal injections of NGF or BDNF, respectively (Morse et al. 1993). Administration of these neurotrophins via

intracerebroventricular injection also prevents cholinergic cell death (Morse et al. 1993; Knusel et al. 1992), but the actions of BDNF are diminished, presumably due to the high concentration of truncated BDNF receptors (trkB) expressed by the ependymal cells lining the ventricles (Yan et al. 1994). In addition to septohippocampal neurons, several studies have recently shown that motor neuron death can also be prevented (Yan et al. 1992; Oppenhein et al. 1992; Sendtner et al. 1992) and differentiated functions restored (Chiu et al. 1994) by BDNF. These effects were developmental stage and region-specific such that, although BDNF administration had beneficial effects on neuron survival in all cases, the magnitude of these effects varied. Whereas BDNF mediates the survival of transected cholinergic neurons, effects on transected dopaminergic neurons have not yet been demonstrated (Knusel et al. 1992; Lindsay et al. 1994), suggesting that protective actions are also neuronal phenotype-specific.

I-6.b. Neuroprotective Actions of BDNF on Dopaminergic Neurons

Investigations of the effects of BDNF on dopaminergic neurons have utilized both tissue culture models and classical neurodegeneration models carried out in animals. Hyman and colleagues (1991) observed that treatment of neuron-enriched E14 mesencephalic cultures with BDNF greatly increased their survival times. Whereas dopaminergic neurons in control cultures were reduced to 25% of the original number by day 8 in culture, neurons in BDNF treated cultures were not significantly different from controls at this point in time. These investigators went on to demonstrate that, in addition to its trophic effects on dopaminergic neurons, BDNF was also capable of protecting them from degeneration imposed by MPP+ treatment. While the number of dopaminergic cells in control cultures was

decreased approximately 4-fold by treatment with MPP^+ for 48 hours, this decrease was abolished in BDNF treated cultures. The authors postulated that the neuroprotective effects of BDNF were due directly to BDNF, but since the cultures were not entirely devoid of glial cells, an indirect action could not be ruled out. Since the original report of Hyman and colleagues, several other investigators have evaluated the ability of BDNF to protect cultured dopaminergic cells from other neurotoxins, as well as neuroprotective actions *invivo*.

Beck et al. (1992) measured the survival and maintenance and of dopamine uptake in fetal dopaminergic neurons after administration of MPP^+. The results were congruent with those of Hyman and coworkers (Hyman et al. 1991), indicating that pretreatment of these cells with BDNF decreases their susceptibility to MPP^+ toxicity. Using other dopaminergic neurotoxins, such as 6-OHDA or 6-hydroxyDOPA, a naturally occuring DOPA metabolite, other investigators have also shown that BDNF has neuroprotective actions on cultured dopaminergic neurons (Spina et al. 1992; Fadda et al. 1993; Skaper et al. 1993). In a study designed to determine the mechanism of the protective actions of BDNF, Spina et al. (1992) compared the actions of BDNF on E14 cultures treated with MPP+ or 6-OHDA to those of NGF. Exposure to either of these neurotoxins resulted in a loss of 70-80% of tyrosine hydroxylase positive neurons that was significantly attenuated by pretreatment with BDNF, but not NGF. Since parallel experiments showed that BDNF increased the activity of glutathione reductase by 100% in the dopaminergic cell line SH-SY5Y, the authors suggested that the neuroprotective actions of BDNF might involve reduction of oxygen free radicals (Spina et al. 1992). As opposed to the results demonstrating neuroprotective actions of BDNF on cultured dopaminergic neurons, the results of similar investigations conducted *invivo* are

still controversial. Whereas Lapchak and colleagues (Lapchak et al. 1993) and Beck et al. (1992) reported that BDNF had no protective effects on axotomized nigrostriatal dopaminergic neurons, Lindsay and colleagues (Lindsay et al. 1994) found that pretreatment with BDNF or NT-3 can ameliorate the pathological effects of striatal infusions of 6-OHDA.

I-7 Glial Cell Line-Derived Neurotrophic Factor

I-7.a. Overview

It is clear that BDNF is capable of promoting the survival of dopaminergic neurons (Knusel et al. 1991; Hyman et al. 1991) and of protecting these neurons from degeneration induced by neurotoxins (Hyman et al. 1991; Spina et al. 1992; Lindsay et al. 1993, section I-6.b). However, a more recently discovered trophic factor, glial cell line-derived neurotrophic factor (GDNF, Lin et al. 1993), has all of the trophic and neuroprotective actions of BDNF on dopaminergic neurons and has also been shown to reverse the effects of dopaminergic neurotoxins *in vivo* (Hoffer et al. 1994; Gash et al. 1995; Tomac et al. 1995a). GDNF is the most potent dopaminotrophic factor yet discovered, requiring lower doses than BDNF to completely prevent cell loss after striatal 6-OHDA injections (Sauer et al. 1995). Moreover, GDNF has potent protective actions on axotomized dopaminergic neurons *in vivo* at far lower doses than are required for protection by BDNF (Beck et al. 1995). GDNF also exerts its actions on a more discrete subset of CNS neurons than BDNF having, for example, no effects on nigral GABAergic neurons (Lin et al. 1993). For the reasons listed above, GDNF is a promising candidate for the treatment of Parkinson's disease and since the mechanisms governing GDNF gene

expression are poorly understood, the present work addressed these regulatory mechanisms using the C6 glioma cell model.

I-7.b. General Characteristics of Glial Cell Line-Derived Neurotrophic Factor

Glial Cell Line-Derived Neurotrophic Factor (GDNF) was originally purified out of conditioned medium from rat B49 glial cell cultures based upon its activity in a dopamine uptake bioassay (Lin et al. 1993). It is a distant relative of transforming growth factor β, sharing less than 20% homology with this factor that is highly conserved across the mammalian species examined so far; the human and rat molecules exhibiting 93% homology (Lin et al. 1993). Bioactive GDNF is a 134 amino acid protein that is derived from a 211 amino acid precursor and exists primarily as a disulfide-bonded homodimer. Available data about the rat GDNF gene indicate that two mRNA splice variants exist (Schaar et al. 1993). These transcripts are 700 bp and 622 bp in length and are identical, except for a 78 bp deletion (nucleotides 123 to 200) in the pre-pro region based on the cDNA sequence (Schaar et al. 1994). This deletion resides in a region between a putative secretion signal sequence and the proteolytic cleavage site shown to release mature GDNF (Lin et al. 1993; Schaar et al. 1993; Trupp et al. 1995). Translation of either of these transcripts produces equivalent amounts of the mature protein having equivalent bioactivity, suggesting that an intact pre-pro region is not required for efficient translation (Schaar et al. 1993; Trupp et al., 1995).

I-7.c. Neurotrophic Actions of GDNF

GDNF has prominent effects on the survival and differentiation of fetal

dopaminergic neurons dissected out of the rat midbrain, while having little or no effect on GABAergic or serotonergic neurons in these cultures (Lin et al. 1993). Furthermore, treatment of these cultures with GDNF leads to a 2.5 to 3-fold increase in dopamine uptake, indicating that surviving neurons exhibit enhanced biochemical functions. Johansson et al. (1995) implanted both prenatal and neonatal mesencephalic tissue into the anterior chamber of the eye and measured survival and TH expression capacity after treatment with GDNF. The results indicated that GDNF increases the survival of fetal dopaminergic neurons, enhances TH-positive fiber formation and dopamine release, while only a small increase in the number of dopaminergic terminals was observed.

Trophic actions of GDNF have also been reported for embryonic chick and nodose ganglia in culture (Trupp et al. 1995) and on developing motor neurons (Oppenheim et al. 1995). In this latter report, administration of GDNF to E5 avian spinal motor neuron cultures promoted the survival of approximately 63% of these neurons, as compared to less than 30% survival of control cultures. Another neuronal population involved in motor function, cerebellar purkinje cells, is also sensitive to GDNF as evidenced by the significant increases in survival and differentiation of these cells reported by Mount et al. (1995). Thus, GDNF appears to be a potent growth factor supporting the survival and biochemical health of several populations of CNS neurons during development.

I-7.d. Neuroprotective actions of GDNF

In addition to its trophic actions, GDNF has potent protective actions on both fetal and mature dopaminergic neurons, as well as on motor neurons (Hoffer et al. 1994; Beck et al. 1995; Gash et al. 1995; Li et al. 1995; Oppenheim et al. 1995; Sauer et

al. 1995; Tomac et al. 1995a; Yan et al. 1995). Survival of either population of neurons following axotomy (Beck et al. 1995; Oppenheim et al. 1995; Yan et al. 1995) or of dopaminergic neurons treated with neurotoxins (Hoffer et al. 1994; Gash et al. 1995; Sauer et al. 1995; Tomac et al. 1995) is significantly enhanced by prior treatment with GDNF. Moreover, GDNF has prominent restorative effects on nigral dopaminergic neurons of mice and nonhuman primates previously compromised by N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP, Gash et al. 1995; Tomac et al. 1995) or 6-hydroxydopamine (Hoffer et al. 1994; Sauer et al. 1995). These latter studies are especially compelling, since rejuvenation of dying nigrostriatal neurons by post-lesion application of a neurotrophic factor had not been demonstrated previously.

After unilateral transection of the medial forebrain bundle, an approximately 53% reduction in TH immunoreactivity occurs in the ipsilateral substantia nigra of adult rats (Beck et al. 1995). Repeated injections of GDNF, but not TGF-α, bFGF or NT-4/5, into this region attenuated dopaminergic cell loss, allowing approximately 85% of these neurons to survive. Although BDNF is also capable of protecting nigrostriatal neurons in a similar paradigm (Hagg, 1994), much higher doses were required, suggesting that GDNF has more potent neuroprotective actions than BDNF on degenerating dopaminergic neurons.

Tomac et al. (1995a) and Gash et al. (1995) used similar experimental procedures in mice and primates, respectively, to examine the neuroprotective effects of GDNF on dopaminergic neurons *in vivo*. When this factor was injected over the substantia nigra or into the striatum prior to administration of MPTP to mice, significant protection of TH-positive neurons resulted, especially after the striatal injections (Tomac et al. 1995a). This latter phenomenon is in line with the recent demonstration of retrograde transport of GDNF by nigrostriatal dopaminergic

neurons (Tomac et al. 1995b). When GDNF was injected into the striatum 1 week after the administration of MPTP, significant restoration of dopamine levels in the substantia nigra and striatum was evident, but of lesser magnitude than that seen after GDNF pretreatment (Tomac et al. 1995a). Whether GDNF was injected prior to or after MPTP, several indices of motor function, including locomotion, rearing and motility, were also significantly improved. Similar to the results of Tomac and coworkers, Gash et al. (1995) reported both protective and restorative effects of GDNF in MPTP treated primates using both behavioral and neurochemical measures.

In their investigation of the neuroprotective effects of GDNF on 6-OHDA-induced dopaminergic neuron degeneration in rats, Hoffer et al. (1994) reported that intranigral injections of GDNF carried out 4 weeks after unilateral lesions significantly reduced aberrant rotational behavior and restored dopamine levels in the ipsilateral substantia nigra. Similarly, Sauer and coworkers (Sauer et al. 1995) utilized a modified 6-OHDA injection procedure (Sauer et al. 1994), whereby intrastriatal injections of this neurotoxin lead to progressive degeneration of dopaminergic neurons over a period of about 4 weeks, to evaluate the effects of intranigral GDNF administration. These investigators found that the 65% loss of dopaminergic neurons projecting to the striatal injection site observed 4 weeks after lesioning could be abolished by chronic infusions of GDNF. Moreover, a single large dose of GDNF administered 1 week after 6-OHDA afforded a partial protection of these neurons.

I-8 Parkinson's Disease

I-8.a. Rationale for the Treatment of Parkinson's Disease with BDNF and/or GDNF

Current treatment strategies for neurodegenerative disorders, such as Parkinson's disease, are centered on transmitter replacement. Parkinson's disease is caused by degeneration of the nigro-striatal dopaminergic neurons originating in the zona compacta of the substantia nigra. In patients with Parkinson's disease, this degeneration progresses over many years, leading to characteristic symptoms including tremor, rigidity, bradykinesia, postural instability and eventual total motor incapacitation.

Treatment of Parkinson's disease consists of attempts to replace the dopamine lost as nigro-striatal dopaminergic neurons degenerate. Exogenous levodopa (L-Dopa) enters remaining neurons where it is metabolized to dopamine via aromatic L-amino acid decarboxylase. It can then be packaged and released to act as a transmitter on postsynaptic cells in the striatum. Unfortunately, the diminished capacity of the dying presynaptic dopaminergic neurons to synthesize dopamine coupled with the complex nature of the response of the postsynaptic cells to fluctuations in the concentrations of dopamine in the synapse commensurate with L-Dopa treatment leads to progressive motor complications in Parkinson's disease patients (Mouradian and Chase, 1988). Moreover, these complications appear to follow fluctuations in L-Dopa concentrations in the blood (Mouradian et al. 1987), and are often as debilitating as the untreated disorder. In both Parkinson's disease and other neurodegenerative disorders such as Alzheimer's disease, transmitter replacement therapies treat only the symptoms of the respective disease and not the underlying disease process.

Since BDNF and GDNF have important trophic, protective and even restorative actions on dopaminergic neurons, they are prominent candidates for treatment of Parkinson's disease. Theoretically, nigral dopaminergic neuron cell death occurring in Parkinson's disease might be a consequence of inadequate exposure to neurotrophic factors due to the breakdown of paracrine, autocrine or target-derived mechanisms. Alternatively, the disease process may generate destructive oxygen metabolites that could be neutralized by these factors *in situ* as discussed in section I-8.b. Thus, timely upregulation of BDNF and/or GDNF gene expression in the substantia nigra or striatum could theoretically ameliorate the symptoms of Parkinson's disease. Identification of cis-acting DNA elements involved in the activation or repression of these genes should therefore allow the development of genomic strategies to modulate their expression for therapeutic purposes.

I-8.b. The Pathogenesis of Parkinson's disease

Although the mechanism responsible for the dopaminergic neuron degeneration in Parkinson's disease has not yet been elucidated, it may be similar to one or more of the mechanisms involved in neuronal death induced by toxic L-Dopa metabolites or 6-OHDA (Chieuh et al. 1993). When 6-OHDA is injected into the zona compacta of the SN or the medial forebrain bundle, greater than 90% of the dopaminergic cells die within 1 week (Jeon et al. 1995). Injection of 6-OHDA into the striatum, which contains the terminals of the nigro-striatal cells also results in cell death, but follows a longer time course, which is more congruent with the extended progression of the disease in humans (Bernheimer et al. 1973; Jeon et al. 1995). 6-OHDA is susceptible to oxidation at physiological pH and subsequent

creation of hydrogen peroxide (H_2O_2), superoxide radical (O_2-) and hydroxyl radical (OH-) is believed to initiate cell death (Cohen and Heikkila, 1974). Because nigrostriatal 6-OHDA lesions mimic the pathological consequences of advanced Parkinson's disease, this procedure has been extensively utilized as a model for evaluating the effects of dopaminergic cell loss on the functioning of the basal ganglia.

Another important animal model of Parkinson's disease is centered on the neurotoxic actions of N-methyl-4-phenylpyridinium ion (MPP^+), the toxic metabolite of MPTP. This compound is actively internalized by dopaminergic neurons and rapidly accumulates in mitochondria (Ramsay et al. 1986) where it inhibits NADH-linked electron transport leading to cell death. Although this mechanism of action is distinct from that of 6-OHDA, administration of MPTP produces a Parkinson's disease-like syndrome in primates and humans (Burns et al. 1983) and has therefore been utilized to assess the neuroprotective qualities of a multitude of potential therapeutic agents, including the neurotrophins (Lindsay et al. 1993). Moreover, MPP^+ also causes a dose-dependent increase in OH^- formation in the striatum, which is thought to be involved in dopamine oxidation producing neuromelanin in the zona compacta of the substantia nigra (Chiueh et al. 1993). Thus, toxic metabolites of MPTP evoke dopaminergic neuron death via multiple mechanisms that may also be operating during the progression of Parkinson's disease.

Since the common denominator in animal models of Parkinson's disease is the presence of elevated levels of oxygen free radicals, it is possible that neurotrophins ameliorate dopaminergic neuron degeneration by reducing the formation of these highly destructive molecules. Accordingly, Spina et al. (1992)

reported that pretreatment of the catecholaminergic cell line SH-SY5Y with BDNF abolished the rise in oxidized glutathione caused by 6-OHDA, and increased levels of glutathione reductase activity. In addition, Cheng and Mattson (1994) provided evidence that BDNF protects hippocampal neurons from hypoglycemia by promoting calcium homeostasis and suggested that free radicals generated under pathophysiological conditions may be involved in neuronal injury. Since growth factors can enhance free radical neutralizing systems (Zhang et al. 1993), it is possible that BDNF exerts its neuroprotective actions via multiple mechanisms.

I-9 Rationale for the Study of the Transcriptional Mechanisms Involved in the Expression of BDNF and GDNF

The discovery, in 1991, that BDNF was capable of protecting dopaminergic neurons from neurotoxin-induced cell death (Hyman et al. 1991), raised the exciting possibility that dopaminergic neuron degeneration attending the advancement of Parkinson's disease might be retarded or even halted by this factor (Lindsay et al. 1993). Since BDNF has documented protective actions on dopaminergic neurons (section I-6.b), that are unparalled by other members of the neurotrophin superfamily, this factor is an especially strong candidate for the treatment of this disease.

Studies of signal transduction mechanisms responsible for the regulation of BDNF mRNA expression implicate calcium, and to a lesser extent cAMP and phosphoinositol metabolites (section I-5.b). Although these studies have shed considerable light on the intracellular mechanisms involved in insult-induced BDNF mRNA elevations, the link between elevation of second messengers and activation of BDNF gene transcription is unclear. Since calcium influx has been

implicated in the regulation of BDNF gene expression in several cell types (Zafra et al. 1990,1991,1992, see Lindvall et al. 1994 for review) and BDNF can, in turn, regulate neuronal survival by stabilizing intracellular calcium concentrations (Cheng and Mattson, 1994), the role of calcium in the regulation of BDNF gene transcription is an important research topic. The observation that calcium regulates BDNF mRNA expression implies that the BDNF gene may contain one or more calcium responsive promoters. Alternatively, calcium may upregulate the stability of BDNF transcripts, which would also account for reported elevations of BDNF transcripts. To resolve these issues, it is clear that determination of the mechanisms resposible for calcium-induced increases in BDNF transcript levels, including identification of putative calcium-responsive DNA elements in the BDNF gene are important research goals. Once these questions have been answered, novel strategies designed to increase the endogenous production of BDNF for the treatment of Parkinson's disease and other neurodegenerative disorders will be facilitated.

Since the intracellular mechanisms regulating GDNF expression have not yet been addressed, and the neuroprotective actions of this factor rival, or even surpass, those of BDNF, understanding the mechanics of GDNF gene regulation is also of crucial importance for the development optimal therapeutic stategies for the treatment of neurodegenerative disorders.

In this dissertation, the contributions of alternative promoters to the regulation of BDNF mRNA expression in different regions of the rat brain, and the signal transduction pathways involved in the activation of BDNF and GDNF gene transcription were studied.

Chapter II Specific Aims

II-1 Overview

The project was carried out in three phases. In phase 1, 5′ untranslated exons upstream of the coding exon of the rat BDNF gene were identified. In phase 2, the differential expression of these exons in the basal ganglia and other brain regions, as well as signal transduction pathways involved in BDNF and GDNF mRNA expression, were investigated. In addition, possible compensatory BDNF mRNA production by striatal cells after chronic dopaminergic denervation was evaluated. In phase 3, the 5′ flanking region of a one of the BDNF alternate exons (exon 1e) was analyzed using a tissue culture model developed in phase 2.

II-2 Phase 1

Previous reports from two different laboratories have shown that the rat BDNF gene is comprised of at least four 5′ untranslated exons that are each spliced directly to the exon containing the structural (coding) BDNF sequence (Timmusk et al. 1993; Nakayama et al. 1994). Since both of these groups identified these exons in cDNAs made from RNA isolated from rat hippocampus, phase 1 work focused on another brain region known to express high levels of BDNF mRNA, the cerebellum. The rationale was that if BDNF transcription proceeds from alternative promoters linked to brain region specific 5′ exons, focusing on just one region precludes the discovery of important 5′ exons expressed in other regions. Thus, the sequences of BDNF 5′ exons expressed in the cerebellum were compared to published exon sequences and a list of independent 5′ exons was compiled.

II-3 Phase 2

In phase 2, semi-quantitative PCR was used to measure the expression of each BDNF 5′ exon across brain regions, including portions of the basal ganglia (substantia nigra and striatum), as well as hippocampus and cerebellum. These experiments were designed to estimate the relative contribution of each of the putative alternative promoters controlling expression of the individual 5′ exons to the total BDNF mRNA pool for each of the regions examined. Since the GDNF gene promoter has not yet been characterized, and the distribution of GDNF mRNA has been reported (Schaar et al. 1993; Schaar et al. 1994; Choi-Lundberg and Bohn, 1995; Trupp et al. 1995), studies of GDNF focused on regulatory mechanisms only. To identify signal transduction pathways regulating BDNF and GDNF mRNA expression in rat brain cells, a tissue culture model was developed. Although, several cell lines were found to express BDNF or GDNF, only the C6 glioma cell line expressed both rat BDNF and rat GDNF. Thus, phase 2 experiments focused on gene regulatory mechanisms and signal transduction pathways involved in expression of these neurotrophic factors in C6 cells. Other phase 2 experiments evaluated potential changes in BDNF gene expression in the striatum resulting from long-term dopaminergic denervation imposed by injections of 6-hydroxydopamine into the medial forebrain bundles of rats. The rationale for these latter experiments was that degenerating nigrostriatal neurons might elicit a neuroprotective response by target cells in the striatum which might include upregulation of BDNF expression in this region.

II-4 Phase 3

Considering the limitations of delivering an exogenous protein such as BDNF or GDNF into the brain at therapeutic concentrations, devising means of augmenting the expression of the endogenous gene coding for these factors have clear advantages. Understanding the regulatory processes leading to the tissue-specific expression of these neurotrophins is an important step in this process. In phase 3, the 5′ flanking region upstream of BDNF exon 1e was cloned and transcriptional regulatory elements were evaluated via 5′ deletion analysis in transient transfection assays. This region was selected because exon 1e-containing transcripts were the most abundant BDNF transcripts in C6 cells, as determined by preliminary experiments. Exon 1e promoter regions that were active in C6 cells were identified and screened for known *cis* -acting DNA elements by computer-aided sequence analysis.

Chapter III Experimental Design and Methods

III-1 Rapid Amplification of 5′ cDNA Ends (5′ RACE)

Rat cerebelli were purchased from Zivic Miller (Zelienople, PA), homogenized with a Polytron homogenizer (Brinkman Instruments, Westbury, NY) and total RNA was isolated using CsCl density gradient centrifugation (Sambrook et al. 1989). Poly(A)$^+$ RNA was purified by oligo d-T columns (Stratagene, La Jolla, CA). Cloning of the 5′ ends of BDNF transcripts expressed in the cerebellum was accomplished using the 5′ RACE (Rapid Amplification of 5′

cDNA Ends) System kit (Gibco/BRL, Grand Island, NY). Briefly, 0.5 ug Poly(A)$^+$ RNA was combined with 2 pmol of the BDNF-specific primer RT1 (5'-TGCAACCGAAGTATGAA-3', Table 1) in reaction buffer, heated to 80^{o}C and cooled on ice. Reverse transcriptase (SuperScript, Gibco/BRL) was added and the mixture was incubated at 42^{o}C for 30 minutes. The RNA template was then degraded by RNase H treatment (55^{o}C, 10 minutes) and the primer removed by ultrafiltration (Amicon, Beverly, MA). A poly-C tail was added to the cDNAs using terminal deoxynucleotidyl transferase, followed by inactivation of the enzyme at 65^{o}C. The poly-C tailed cDNA pool was amplified by PCR in the presence of an anchor primer containing a stretch of poly-G coupled to a directional cloning linker (supplied in the kit), and the BDNF specific nested primer (5'-GCCTTCATGCAACCGAAGTA-3', Figure 3) that also contained a directional cloning linker. Forty cycles of PCR (95^{o}C, 1 minute; 54^{o}C, 1 minute; 72^{o}C, 2 minutes) were performed, the products were ligated into the directional cloning vector pAMP1, (Gibco/BRL) and the resulting plasmids were used to transform E. Coli. Two separate rounds of the 5' RACE procedure were conducted, producing approximately 2500 bacterial colonies. Clones harboring BDNF cDNAs were identified by colony hybridization using the ^{32}P labeled oligonucleotide P8, (5'-AACCATAGTAAGGAAAAGGATGGTCAT-3') which is specific for the 5' end of the published BDNF sequence (Table 1, Figure 3). Seventy positive clones were isolated and sequenced using the standard double-stranded dideoxy chain termination method (Sequenase, U.S. Biochemicals, Cleveland, Ohio).

Table 1. The sequence of each of the primers used for RT and PCR is listed with corresponding target genes and nucleotide positions. BDNF primer positions are relative to the first ATG of the rat BDNF sequence (Bishop et al., 1994; Maisonpierre et al., 1990), designated +1 here. GDNF primer positions are according to the mRNA sequence submitted to Genbank by Lin and coworkers (Lin et al., 1993), accession number L15305. Cyclophilin primer positions are according to the published cDNA sequence (Danielson et al., 1988).

Table 1 Nucleotide sequences of RT-QPCR primers

Gene	Primer	Nucleotide Position	Sequence (5′ - 3′)	Reference
GDNF	RT1	439 to 421	CAAGTCAGTGACATTTAAG	Lin et al., 1993
	P1 (sense)	46 to 71	TAAGATGAAGTTATGGGATGTCGTGG	Lin et al., 1993
	P2 (antisense)	409 to 386	TAAGACGCACCCCCGATTTTTGCC	Lin et al., 1993
BDNF	RT2	278 to 262	GTGTACAAGTCCGCGTC	Maisonpierre et al. 1990
	P3 (coding exon, sense)	94 to 116	TTGGCCTACCCAGCTGTGCGGAC	Maisonpierre et al. 1990
	P4 (coding exon, antisense)	214 to 190	CTCTTCGATCACGTGCTCAAAAGTG	Maisonpierre et al. 1990
	P5 (exon 1b, sense)	-214 to -190	GGTGGATGAGAGTTGAAGCTTGCG	Bishop et al., 1994
	P6 (exon 1d, sense)	-236 to -214	GTGCGAGTATTACCTCCGCCATG	Bishop et al., 1994
	P7 (exon 1e, sense)	-78 to -55	CGTGACAACAATGTGACTCCACTG	Bishop et al., 1994
	P8 (5′ exons, antisense)	27 to 1	AACCATAGTAAGGAAAAGGATGGTCAT	Bishop et al., 1994
Cyclophilin	RT3	480 to 463	CCCAAAACGCTCCATGGC	Danielson et al., 1988
	P9 (sense)	151 to 174	CGTGCTCTGAGCACTGGGGAGAAA	Danielson et al., 1988
	P10 (antisense)	410 to 386	TCCAGCCACTCAGTCTTGGCAGTGC	Danielson et al., 1988

Figure 3. Partial nucleotide sequences of alternate 5′ exons in the rat BDNF gene. Exons 1c, 1d and 1e found in the present investigation are identical to previously reported exons II, III and IV, respectively[20]. Exon 1a is a novel BDNF 5′ exon and exon 1b corresponds to previously reported exon I[20], which was not represented in the RACE clones obtained in the present investigation. The authenticity of all five of these exons was confirmed by sequencing RT-PCR products using RNA from rat cerebellum and each was shown to be independently spliced to the common coding exon (shown on the right side of the Figure). Sequences of antisense primers used for reverse transcription (RT1) and PCR (P8) are indicated with arrows and the sense primers used for confirmation of the 5′ RACE clones are underlined, as is the *Dde* I site in exon 1a. Numbering is relative to the major translation start site, labeled +1.

1a

-164

TTTTTTTTAATTTACCCCTTTCTATTTTCCCTCCCCG

AGAGTTCCGGGCTCTGGCTTGGAGGGCTCCTGCTTTCTCAAGGGAAGGGGAG

CCGCTGAGACTGCGCTCCACTCCTGCCGGGCTGGATGCTTCATTGAGCCCAG **TTCCACCAGGTGAGAAGGGTGATGACCATCCTTTTCCTTACTATGGTTATTTCATACTTCGGTTGCATGAAGGC**

+1

P8 NESTED PRIMER

1b

-659

TAAAGCGGTAGCCGGCTGGT

GCAGGAAAGCAACAAGTTCCCCAGCGGTCTTCCCGCCCTAGCCTGACAAGCC

GAAGCTTTTCTTACCTGGCGACAGGGAAATCTCCTGAGCCGAGCTCATCTTT

GCCACAGCCCCAGGTGTGACCTGAGCAGTGGGCAAAGGAGCGGCGTGCAAAT

TGGATTATTTGTATGGGGGTACTCTGAAACTCCCTCACTTTTTCTGGGAACT

TTTTGTGCTAGGGCGCAGTGACAGGCGTTGAGAAAGCTGCTTCAGGAAACGC

CCGCTATATAGCAGGGCAGTTGGACAGTCATTGGTAACCTCGCTCATTCATT

AGAATCACGTAAGAACTCAAAGGGAAACGTGTCTCTCAGAATGAGGGCGTT

TGCGTAAATCTATAGGTTTTTCAACATCGATGCCAGTTGCTTTGTCTTCTGT

AATCGCCAAGGTGGATGAGAGTTGAAGCTTGCGATATTGCTTTGGGTTATTA

GATTCATAAGTCACACCAAGTGGTGGGCGATCCACTGAGCAAAGCCAACTT

CTCACATGATGACTTCAAACAAGACACATTACCTTCCAGCATCTGTTGGGG

AGACGAGATTTTAAGACACTGAGTCTCCAGGACAGCAAAGCCACAATG **TTCCACCAGGTGAGAAGGGTGATGACCATCCTTTTCCTTACTATGGTTATTTCATACTTCGGTTGCATGAAGGC**

+1

P8 NESTED PRIMER

1c

-127

GGAATAG

ACTCTTGGCAAGCTCCGGGTTGGTATACTGGGTTAACTTTGGGAAATGCAAG

TGTTTATCTCCAGGATCTAGCCACCGGGGGTGGTGAAGCCGCAAAGAAG **TTCCACCAGGTGAGAAGGGTGATGACCATCCTTTTCCTTACTATGGTTATTTCATACTTCGGTTGCATGAAGGC**

+1

P8 NESTED PRIMER

1d

-259

AAATGGAGCTTCTCACTGAAGGCGTGCGAGTATT

ACCTCCGCCATGCAATTTCCACTATCAATAATTTAACTTCTTTGCTGAAGA

ACAGGAGTACATATCGGCCACCAAAGACTCGCCCCCCTCCCCCTTTTAACTG

AAGAGAAGGGGAAATATATAGTAAGAGTCTAGAACCTTGGGGACCGGTCTT

CCCCAGAGCAGCTGCCTTCATGTTTACTTTGACAAGTAGCGACTGAAAAAG **TTCCACCAGGTGAGAAGGGTGATGACCATCCTTTTCCTTACTATGGTTATTTCATACTTCGGTTGCATGAAGGC**

+1

P8 NESTED PRIMER

1e

-366

AATCGAAGCTCAACCGAAGAGCTAAATAATGTCTP

GACCCCAGTGCCTGGCGCTGGCTGAGCTCTGGGTGCCCGCCGCTGCCGCCGCG

CCGGGGGGCACCCGCTGGCTGGCTGTCGGCACGTGTCCCCATTGCGCCCGGAC

TCCCCGGCTTGGAGAAGGAAACGCCTGGGGCGGCGCGCCACCTCCGCCTGGC

AGGCTTTGATGAGACCGGGTTCCCTCAGCTCGCCACCGCTGCTTTGGGGCAG

ACGAGAAAGCGCACGGGGCCCAGGGCAGGGCGCAGGGACCAGGAGCGTGAC

AACAATGTGACTCCACTGCCGGGGATCCGAGAGCTTTGTGTGGACCCTGAG **TTCCACCAGGTGAGAAGGGTGATGACCATCCTTTTCCTTACTATGGTTATTTCATACTTCGGTTGCATGAAGGC**

+1

P8 NESTED PRIMER

III-2 Preparation of Oligonucleotide Primers

Oligonulceotides for use in 5'-RACE procedures and for semi-quantitative PCR were made using an Applied Biosystems DNA synthesizer (Model 394) and purified by polyacrylamide gel electrophoresis (Sambrook et al. 1989). The sequence of each primer utilized, its binding site within the target gene and appropriate references is shown in Table 1. Primers RT1 and RT2 were designed to synthesize BDNF cDNAs, while RT3 and RT4 were designed to synthesize GDNF and cyclophilin cDNAs, respectively. Primers P1 and P2 were used to amplify a 120 bp portion of the BDNF gene coding region; primers P3 - P7 were individually paired with primer P8 to amplify BDNF exons 1a, 1b, 1c, 1d and 1e (Bishop et al. 1994), respectively; primers P9 and P10 were used to amplify a 364 bp portion of the rat GDNF gene; and primers P11 and P12 were used to amplify a 260 bp portion of cyclophilin cDNA. All PCR primer sets had melting temperatures ($T_{m)}$ of approximately 78^{o}C and were designed to produce PCR products that span introns whenever possible, including all five sets targeting alternate BDNF exons. This ensures that correctly sized PCR products result from cDNA and not from genomic DNA. In addition, the T_m of each primer used in reverse transcription (RT) reactions was lower than the annealing temperature used in subsequent PCR so that participation of these primers in the PCR was kept to a minimum. Melting temperatures of primer sets targeting the BDNF, GDNF and cyclophilin coding exons were also set to approximately 78^{o}C, but did not produce intron-spanning products.

III-3 Semi-quantitative PCR

Semi-quantitative PCR was performed using a modification of the procedure reported by Murphy et al. (1990). For studies of BDNF mRNA expression in the rat brain, total RNA was isolated from rat substantia nigra, striatum, hippocampus and cerebellum using RNAzol B (Tel-Test, Inc). For investigation of the roles of signal transduction pathways in regulation of the BDNF and GDNF mRNA expression in C6 cells, RNA isolation and RT-PCR procedures developed to measure these transcripts in the rat brain were applied. However, annealing temperatures and numbers of PCR cycles were sometimes different to optimize quantification. These parameters, as well as concentration-response curves resulting from RT-PCR analysis of C6 cell RNA, are presented in the Results. In standard semi-quantitative PCR assays, aliquots containing 1 μg of total RNA in 20 μl were subjected to RT using SuperScript II (Gibco/BRL). Separate RT reactions included 10 pmol of either the BDNF-specific primer (RT2) or the GDNF-specific primer (RT3). In addition, all reactions contained 10 pmol of primer RT4 to allow measurement of cyclophilin transcripts as an internal control.

Following completion of RT reactions, samples were heated to 95^{o}C for 5 minutes to denature the reverse transcriptase and placed on ice or frozen at -30^{o}C until analyzed by PCR. Serial dilutions of the RT reaction product (cDNA) were subjected to semi-quantitative PCR using conditions designed to amplify either BDNF, GDNF or cyclophilin transcripts for construction of standard curves. BDNF and cyclophilin primer sets were used at 5 and 10 pmol/reaction, respectively, while the GDNF primer set was used at 7.5 pmol/reaction.

In a typical 50 μl reaction, cDNA, *Taq* polymerase buffer, deoxynucleotide

triphosphates (0.5 mM of each dNTP) and a PCR primer set were combined with 0.33 pmol (1-2 x 10^6 cpm) of ^{32}P-labeled antisense PCR primer and 2.5 units of *Taq* DNA polymerase (Boehringer Manneheim, Indianapolis, IN), overlaid with mineral oil and placed in a thermal cycler (MJ Research, Watertown, MA). For determination of BDNF and cyclophilin mRNA levels, PCR reaction tubes were heated for 5 minutes at 80^{o}C followed by 30 or 35 cycles of the progression: 95^{o}C, 1 minute; 60^{o}C, 1 minute; 72^{o}C, 1 minute. GDNF transcript levels were determined in the same manner using 30 cycles of PCR, except that the annealing temperature was 64^{o}C. Labeled PCR products and appropriate size markers were separated in 2% or 2.5% agarose gels and stained with ethidium bromide. Excised gel sections containing ^{32}P-labeled PCR products were placed in scintillation vials and radioactivity was counted. Optimal conditions for the semi-quantitative PCR assay were established by construction of cDNA concentration-percent incorporation curves shown in the Results section. In addition, each sample was subjected to parallel analysis omitting Superscript II enzyme to evaluate potential contamination with genomic DNA. RNA samples in which a genomic DNA signal was detected, as evidenced by production of a visible band despite the omission of Superscript II, were discarded.

III-4 Confirmation of BDNF Exon 1a Transcripts

Confirmation of BDNF exon 1a transcripts was accomplished by restriction enzyme analysis of RT-PCR products and by northern blot analysis using both exon 1a and BDNF coding exon-specific probes. For restriction enzyme analysis, total RNA was extracted from rat hippocampus and subjected to RT-PCR as described in

section III-3. The resulting product was digested with *Dde* I, separated in a 10% nondenaturing polyacrylamide gel and the resulting bands were visualized by ethidium bromide staining. For confirmation of exon 1a by northern blot analysis (Sambrook, 1989), separate blots containing 12-15 ug per lane of poly(A)$^+$ RNA extracted from rat hippocampus (purified using poly(A)+ quik columns from Stratagene) were probed with BDNF exon 1a oligonucleotide probes or with a probe targeting the BDNF coding exon. After stringent washing conditions (0.2X SSC, 55-65^{0}C) the blots were dried, exposed to x-ray film (XAR-2, Kodak) and the resulting autoradiograms were compared.

III-5 Nigrostriatal Tract Lesions

In collaboration with Kai-Xing Huang and Judith R. Walters, NPS, ETB, NINDS, NIH, three sets of male Sprague-Dawley rats received unilateral 6-hydroxydopamine (6-OHDA) injections (10 µg per rat) into the right medial forebrain bundle using the following stereotaxic coordinates: 4.4 mm posterior to bregma, 1.3 mm lateral to midline and 7.8 mm ventral to the dural surface. After a 1 week recovery period, the extent of the ipsilateral lesion was evaluated by injection of apomorphine (0.05 mg/kg, s.c.) followed by assessment of contralateral (counterclockwise) rotations. Animals with behaviorally confirmed nigrostriatal lesions were sacrificed following 2 week, 4 week or 3-6 month survival times. Striata from both 6-OHDA lesioned and unlesioned sides of brains were dissected on ice, sonicated in RNAzol B (Tel-Test, Inc) and an aliquot of the aqueous phase was diluted in ice cold sodium acetate buffer (pH 5.0) for measurement of dopamine levels by high-performance liquid chromatography with electrochemical detection.

Total RNA was extracted from the remaining sample and subjected to semi-quantitative RT-PCR using BDNF coding exon primers (P1 and P2).

III-6 Cell Culture and Treatment Conditions

C6 glioma cells were purchased from American Type Culture Collection (Rockville, MD) and maintained in DMEM containing 10% fetal bovine serum. For each experiment, 10 cm plates were seeded with approximately 1 x 10^6 cells each and allowed to grow to confluency (2-3 days). For experiments measuring BDNF and GDNF expression in response to second messenger-inducing agents, cells were rinsed with Dulbecco's phosphate-buffered saline (PBS) and treated with either 0.01% DMSO (vehicle), A23187 (10^{-5} M), forskolin (10^{-5} M) + IBMX (10^{-5} M) or TPA (10^{-7} M) in serum-free DMEM. A23187 is a calcium ionophore that produces large elevations in intracellular calcium; forskolin and IBMX elevate intracellular cAMP by direct activation of adenylate cyclase and by inhibition of phosphodiesterase activity, respectively; and TPA activates protein kinase C (PKC). After a 4 hour incubation period, treatment solutions were removed, the cells were rinsed with PBS and total RNA was isolated using RNAzol B (Tel-Test, Inc).

To investigate transcriptional versus post-transcriptional regulation, cells were treated for 4 hours with 0.01% DMSO or 10^{-5} M A23187 in the presence or absence of actinomycin D (10 μg/ml) and RNA was isolated as described above. All reagents were purchased from Sigma (St. Louis, MO) except forskolin, which was purchased from Calbiochem (San Diego, CA). Total RNA was stored at -80^{o}C until used for RT-PCR assays.

III-7 Transient Transfection Assays

III-7.a. Construction of BDNF Exon 1e Promoter 5′ Deletion Plasmids

The region of the rat BDNF gene extending from nucleotides 857 - 1966 of the exon IV clone described by Timmusk et al., (1993) was cloned by PCR using rat genomic DNA as the template and the following primers:

P33 (5′-GCGTGCGAGTATTACCTCCGCCATGC-3′) and

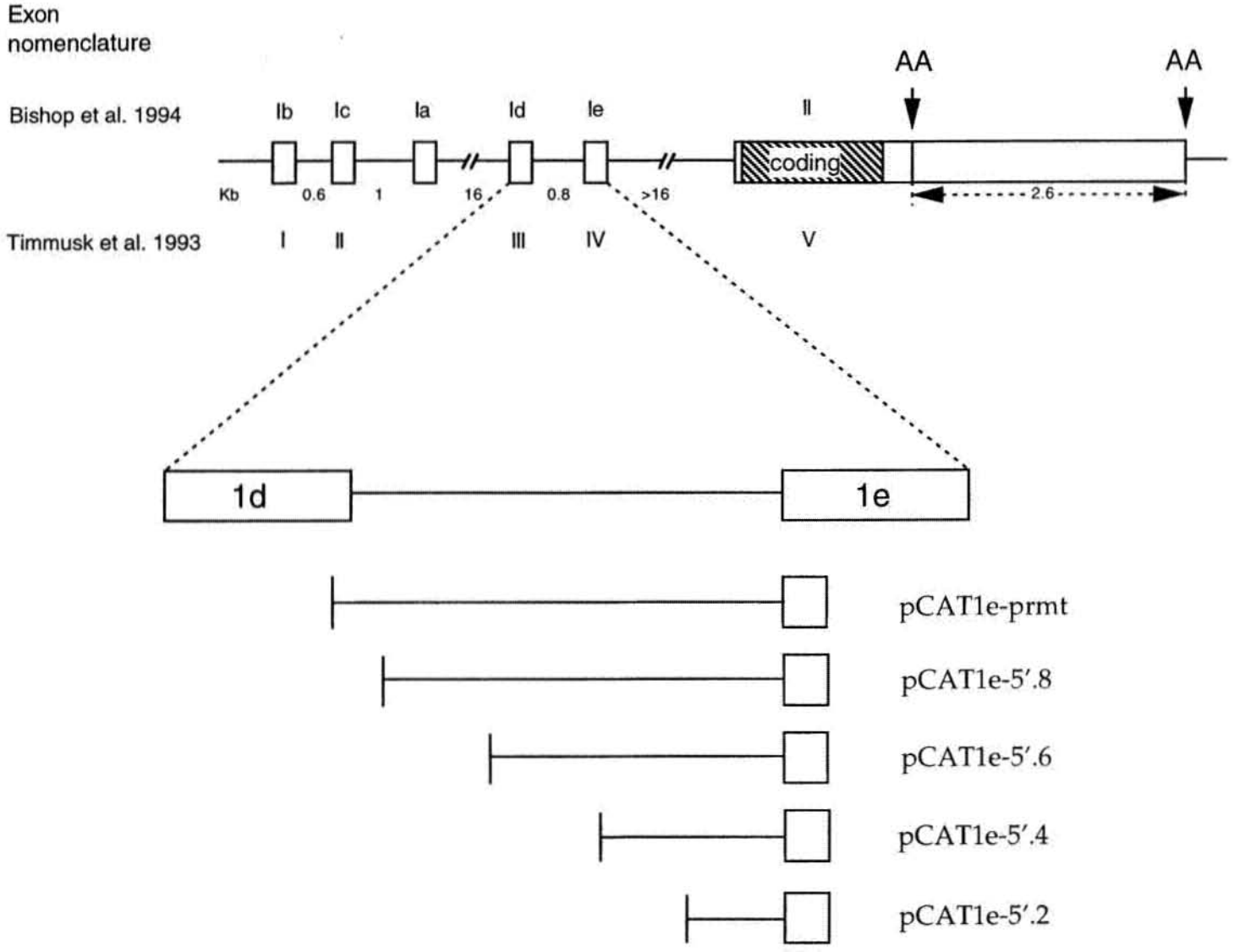
P34 (5′-CAGCGGTGGCGAGCTGAGGGAA-3′)

One ug of genomic DNA was amplified with 40 cycles of PCR, using the procedure described for the semi-quantitative PCR assay, with the annealing temperature set to 60^{o}C. The resulting band of the appropriate size was subcloned into the PCRII vector (Invitrogen, San Diego, CA), yielding the vector termed TA-1e-prmt. The insert was then transferred into the CAT reporter vector pCAT Basic (Promega, Madison, WI), yielding pCAT1e-prmt. Derivatives of this latter plasmid, having sequential 5′ deletions, were then constructed by PCR using the primers shown in Table 2. Primers P44, P45, P46 and P47 were sense primers to which a 5′ *Pst* I site was added and P43 was the antisense primer to which a 5′ *Xba* I site was added. The sense primers were then individually paired with the antisense primer and used to amplify 1 ng aliquots of pCAT-1e.prmt. After 25 cycles of PCR using the procedures described above, the resulting products were gel purified and ligated into the *Pst* I/*Xba* I site of pCAT Basic (Promega), using T4 DNA ligase (Sambrook, 1989). The resulting constructs were termed pCAT1e-5′.8, pCAT1e-5′.6, pCAT1e-5′.4 and pCAT1e-5′.2 to indicate that they contained 5′ deletions of the BDNF 1e promoter region, see Figure 4.

Table 2. The sequences of the PCR primers used to generate BDNF exon 1e 5′ deletion constructs are listed with corresponding nucleotide positions taken from the sequence reported by Timmusk et al. (1993). The antisense primer (P43), which contained a *pst*1 site at the 3′ end, was paired with each of the sense primers, which contained 5′ *xba*1 sites and the resulting PCR products were ligated into *pst*1-*xba*1 cleaved pCAT Basic.

Primer	Position	Nucleotide Sequence (5′ - 3′)
P43	1800 to 1775	GCTCTAGACCAGAGCTCAGCCAGCGCCAGGCAC
P44	1029 to 1055	ACCTGCAGGGAAGCATATGGCTTTAGTGAGGTCTG
P45	1227 to 1251	AACTGCAGCCATAACCCCGCACACTCTGTGTAG
P46	1421 to 1445	AACTGCAGGTCACACTTGTTCCCTCCTACTCTG
P47	1626 to 1650	AACTGCAGCTGAATCCCGGCAAGGAAAAGGCGC

Figure 4. Structure of the rat BDNF gene and exon 1e promoter fragments used to construct CAT reporter vectors. The structure shown at the top of the figure is a compilation of previously described attributes of the rat BDNF gene. The lower portion of the figure lists the exon 1e 5′ deletion constructs used in transient transfection assays. The insert in pCAT1e-prmt is 900 bp in length (nucleotide 901 to 1800, Timmusk et al. 1993) and all others are sequentially shortened versions of this parent construct (see Experimental Design and Methods for details).


Exon
nomenclature
Bishop et al. 1994
Timmusk et al. 1993
Ib
Ic
Ia
Id
Ie
II
AA
AA
coding
Kb
0.6
1
16
0.8
>16
2.6
I
II
III
IV
V
1d
1e
pCAT1e-prmt
pCAT1e-5′.8
pCAT1e-5′.6
pCAT1e-5′.4
pCAT1e-5′.2

The decimals signify the approximate length of endogenous BDNF promoter sequence contained in each of these vectors. For example, pCAT1e-5'.8 contains approximately 800 bp and pCAT1e-5'.2 contains about 200 bp (Figure 4). Each construct, as well as the parent vector, pCAT Basic, was transfected into competent *E.Coli* strain HB101 (Life Sciences) to prepare milligram quantities of each for sequence verification and transient transfection assays.

III-7.b. Transient Transfection of C6 Glioma Cells

C6 cells were transfected with CAT and β-gal constructs using a previously described modification of the calcium phosphate procedure (Bishop and Mouradian, 1993). For each 10 cm dish of cells, a solution of plasmid DNA (10 μg CAT construct + 5 μg ras β-gal) in 0.25 M $CaCl_2$ was slowly added to 2X-Hepes buffered PBS, incubated for 20-30 minutes at room temperature and mixed with an aliquot of C6 cells (approximately 5 X 10^6 cells). After an additional 15 minute incubation period, the cells were added to dishes containing DMEM/10% FBS and incubated overnight in a 4% CO_2 incubator. On the following day, the precipitate was removed, the cells were rinsed with PBS, fresh medium was added and the dishes were placed in a 10% CO_2 incubator. After a 48 hour period, the cells were harvested and processed for β-gal and CAT assays.

III-7.c. β-galactosidase and Chloramphenicol Acetyltransferase Assays

β-gal activity in transfected cells was measured using a standard procedure

(Sambrook et al. 1989). Transfected C6 cells were rinsed with PBS, scraped from the dishes, triturated in 1 ml of PBS and transfered into ice cold 1.5 ml microfuge tubes. The tubes were then centrifuged for 3 minutes at 6000 rpm in a microfuge and resuspended in 1 ml TEN buffer (40 mM Tris-HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl) followed by recentrifugation. The resulting pellet was resuspended in 250 µl of 250 mM Tris-HCl, pH 7.5 and lysed by 3 cycles of freezing on dry ice followed by thawing in a 37°C water bath. The samples were then centrifuged for 10 minutes at 14000 rpm in a microfuge and the supernatants were removed for β-gal and CAT assays. For determination of β-gal activity, 30 µl of supernatant from each sample was added to β-gal reaction cocktail containing 1 mM $MgCl_2$, 0.2 mg of β-gal substrate, o-nitrophenyl-β-D galactopyranoside (ONPG), and 200 µl of 0.1 M sodium phosphate, pH 7.5. Samples were then incubated for 1-4 hours at 37°C and after the development of light yellow color, the reactions were stopped with 500 µl of 1 M Na_2CO_3 and read at 420 nm on a spectrophotometer. The resulting absorbance values were used to normalize CAT ELISA data for transfection efficiency.

The production of CAT by transfected cells was measured using a CAT ELISA kit (Boehringer Mannheim). Briefly, 200 µl aliquots of transfected cell lysates were added to assay plate wells precoated with anti-CAT antibodies and, following several washing steps, anti-CAT antibodies coupled to digoxygenin were added followed by addition of digoxygenin substrate. After a 1-2 hour incubation period, the concentration of bound CAT enzyme was determined by measurement of green reaction product at 405 nm.

III-8 Data Analysis

III-8.a. Semi-Quantitative RT-PCR Data

Concentration-percent incorporation curves for BDNF, GDNF and cyclophilin assays were prepared by plotting concentrations of cDNA included in PCR reactions against amounts of ^{32}P-labeled primer counts incorporated into resultant PCR products. This information was then used to select optimal cDNA concentrations for subsequent QPCR analyses. Curves for all three types of transcripts were generated from C6 cell RNA, while only BDNF and cyclophilin curves were required for analysis of rat brain samples. In experiments employing semi-quanatitative PCR, the concentrations of BDNF and GDNF transcripts were determined relative to concentrations of cyclophilin transcripts in each RNA sample. This was accomplished by dividing percent incorporation of ^{32}P-labeled BDNF or GDNF primer into their respective PCR products by percent incorporation of ^{32}P-labeled cyclophilin primer into cyclophilin products for each sample. Note that approximately 50 times more cDNA was used to measure BDNF transcripts in the hippocampus than was used to measure cyclophilin transcripts in the same region. Thus, counts obtained from QPCR of a hippocampal sample with a BDNF primer set was divided by 50 and the resulting value was divided by the cpm obtained with the cyclophilin primer set for the same sample. QPCR results obtained from other rat brain regions and from experiments conducted on C6 cells also required adjustment, since optimal cDNA input values differed, especially for the BDNF alternate exon transcripts. In all of these assays, input cDNA concentrations determined from concentration-percent incorporation curves were

used and all data were analyzed by ANOVA with post-hoc tests using a commercially available software package (Statview, BrainPower, Inc., Calabase, CA).

III-8.b. CAT Expression Data

In transient transfection assays evaluating the relative strengths of exon 1e promoter segments, absorbance values from CAT assays were divided by β-gal values to control for varying transfection efficiencies across treatment conditions. The resulting ratios were then divided by the ratios obtained after transfection of pCAT Basic, recorded as percent of control and analyzed by ANOVA with post-hoc tests (Statview).

Chapter IV Results

IV-1 Cloning of BDNF Alternate 5′ exons

Complementary DNA copies of the 5′ ends of rat BDNF transcripts were cloned using rat cerebellar poly(A)$^+$ RNA and the 5′ RACE procedure. Of 70 clones sequenced, four independent clones were identified: 1a, 1c, 1d and 1e (Figure 3). The sequence of exon 1b, which was not included among our 5′ RACE clones, is taken from the previously published genomic sequence (Timmusk et al. 1993) in which exons I, II, III, and IV correspond to the present exons labeled 1b, 1c, 1d and 1e, respectively (Figure 4).

The sequence labeled exon 1a, which was discovered in the present investigation, is derived from a novel BDNF 5′ exon that does not have significant homology to sequences reported to Genbank or other DNA sequence databases. As

shown in Figures 1 and 4, it is probably located just downstream of exon 1c (T. Timmusk, personal communication). Restriction analysis of the exon 1a RT-PCR product using *Dde* I produced fragments of approximately 96 and 79 bp (Figure 5), which was predicted by the location of this restriction site in the original RACE clone (Figure 3). To further ensure the authenticity of this novel 5′ exon, poly(A)$^+$ RNA prepared from hippocampus was subjected to northern blot analysis using exon 1a-specific oligonucleotide probes. Results indicated that exon 1a-containing transcripts have gel mobilities that are identical to BDNF transcripts visualized with a probe specific for the coding exon (Figure 6).

To confirm that each of the BDNF 5′ exons shown in Figure 3 is expressed in rat cerebellum, RT-PCR experiments were conducted. Each of the five first exon-specific primers was separately paired with the antisense primer P8 and subjected to PCR using aliquots of RT2-primed cerebellar cDNA as template (see Table 1 for primer sequences). The results showed that each primer set produced a RT-PCR product of the appropriate size that was subsequently proven to be identical to the original 5′ RACE clone by DNA sequencing.

IV-2 Semi-Quantitative RT-PCR Assays

Since individual BDNF 5′ exon transcripts are found in low abundance, especially in the striatum (Maisonpierre et al. 1990; Okazawa et al. 1992), a highly sensitive and reliable semi-quantitative PCR assay was developed to measure the levels of these transcripts in various regions of the rat brain. Products resulting from RT-PCR of BDNF 5′ exons were visualized as single bands in agarose gels and ranged in size from 105 bp (exon 1e product) to 263 bp (exon 1d product).

Figure 5. Confirmation of exon 1a by restriction enzyme analysis. Total RNA was extracted from rat hippocampus and subjected to RT-PCR as described in Chapter II. The resulting product was digested with *Dde* I and separated in a 10% nondenaturing polyacrylamide gel. Lane 1, molecular size standards; lane 2, intact exon 1a RT-PCR product; lane 3, *Dde* I cleaved RT-PCR product.

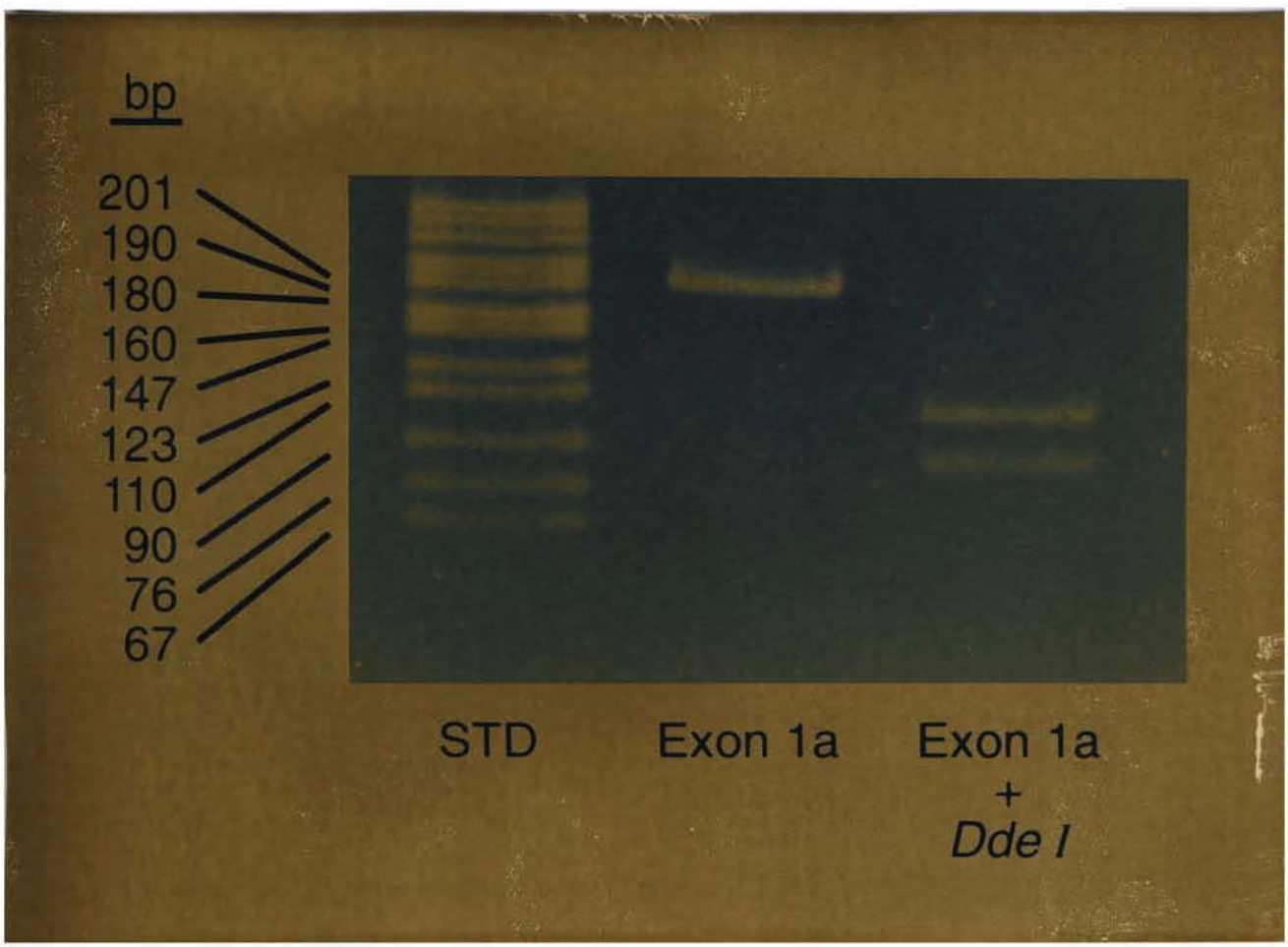
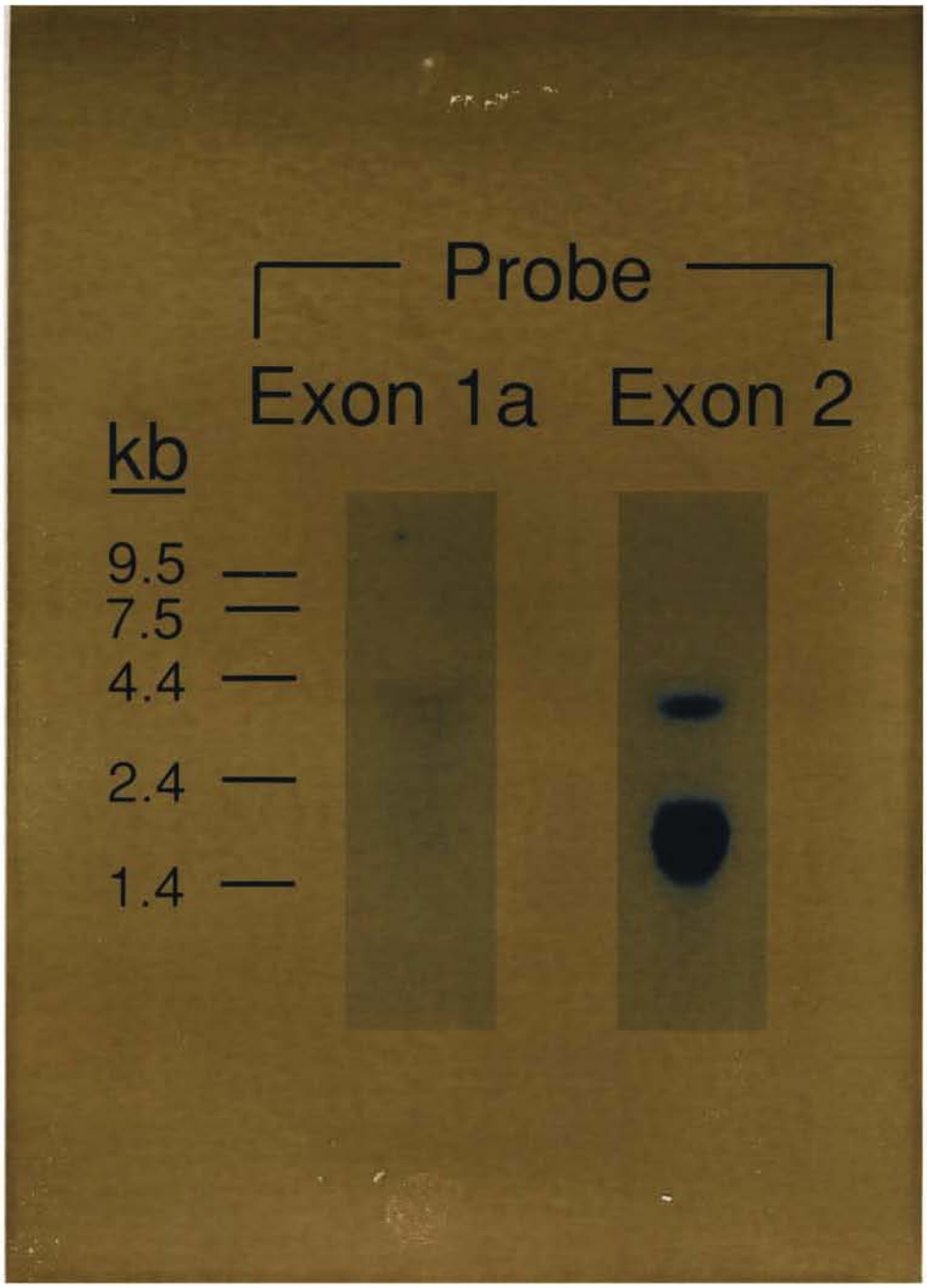

bp
201
190
180
160
147
123
110
90
76
67
STD
Exon 1a
Exon 1a
+
Dde I

Figure 6. Confirmation of exon 1a by northern blot analysis. Separate blots containing 12-15 ug per lane of poly(A)$^+$ RNA extracted from rat hippocampus were probed with 3 separate BDNF exon 1a oligonucleotide probes or with a probe targeting the BDNF coding exon (exon 2, P8). Under stringent washing conditions (0.2X SSC, 55-65^{o}C) all 3 exon 1a probes yielded autoradiographs essentially identical to the one shown in the left-hand panel above, which was obtained using an antisense probe (5'-GGCTCAATGAAGCATCCAGCCCG-3') complementary to nucleotides -46 to -24 relative to the translation start site (see Figure 1a). Mobilities of the 2 species, which are due to alternate polyadenylation sites (Timmusk et al. 1993), were identical to those visualized with the exon 2 probe. The left-hand (exon 1a) bands are the result of a 72 hour exposure, while the right-hand (exon 2) bands required only an 18 hour exposure.


Probe
Exon 1a
Exon 2
kb
9.5
7.5
4.4
2.4
1.4

The cyclophilin RT-PCR product also ran as a single band that was 260 bp in length. To normalize the amount of RNA used in semi-quantitative PCR experiments, replicate aliquots of RNA from each sample were reverse transcribed using a rat cyclophilin primer (RT4, Table 1) and subjected to PCR analysis. Serial dilutions of cyclophilin cDNA derived from rat striatum led to linear decreases in ^{32}P emissions measured in excised PCR bands throughout the range of dilutions used (Figure 7). In all brain regions examined, when greater than 0.8% of the RT reaction was amplified, the concentration-percent incorporation curve was no longer linear and when less than 0.025% of the RT reaction was amplified, ^{32}P emissions approached background levels measured in adjacent blank lanes of the gel.

Based on these results, input cDNA was either 0.2% or 0.4% of the RT reactions for measuring cyclophilin transcript levels in all brain regions examined. Analogous experiments conducted on BDNF 5′ exon cDNAs, using primer sets for each of the five first exons yielded similar results. For example, dilutions of exon 1c cDNA in the range of 2.5% to 20% of RT reactions performed on hippocampal RNA were in the linear portion of the concentration-percent incorporation curve (Figure 8). When greater than 20% of the cDNA was amplified, the curve began to plateau, and when less than 2.5% was amplified, emissions were not significantly different from background. Thus, for measurement of exon 1c transcripts in the hippocampus, 5% of the cDNA from a single RT reaction was used in PCR. Optimal dilutions of input cDNA made from all brain and C6 cell samples were determined in a similar fashion for each of the other BDNF 5′ exons, as well as for GDNF and cyclophilin transcripts, to assure that all measurements were made within the linear segments of the cDNA concentration-percent incorporation curves. Details of the RT-PCR assays applied to C6 cell RNA are presented in section IV-5.a.

Figure 7. Determination of optimal cDNA dilutions for quantitation of cyclophilin transcripts. Total RNA from rat striatum was reverse transcribed using a rat cyclophilin primer (RT4) and serial dilutions of the resulting cDNA were subjected to PCR using a primer set designed to amplify nucleotides 151-450 of the published rat cyclophilin cDNA (P11, P12, Danielson et al. 1988). Input cDNA is indicated as percentage of the original RT reaction volume. One pmol of ^{32}P-labeled antisense PCR primer was added to each tube, the resulting products were excised from 2% agarose gels and counted in a scintillation counter. The obtained counts were converted to a percentage of the total ^{32}P-primer added per PCR tube. Findings are taken from a single representative experiment and are comparable to similar analyses carried out using RNA from substantia nigra, hippocampus and cerebellum. Data points represent means ± S.E.M. (n=3).
INSET is taken from a photograph of a dilution series used to construct the graph.

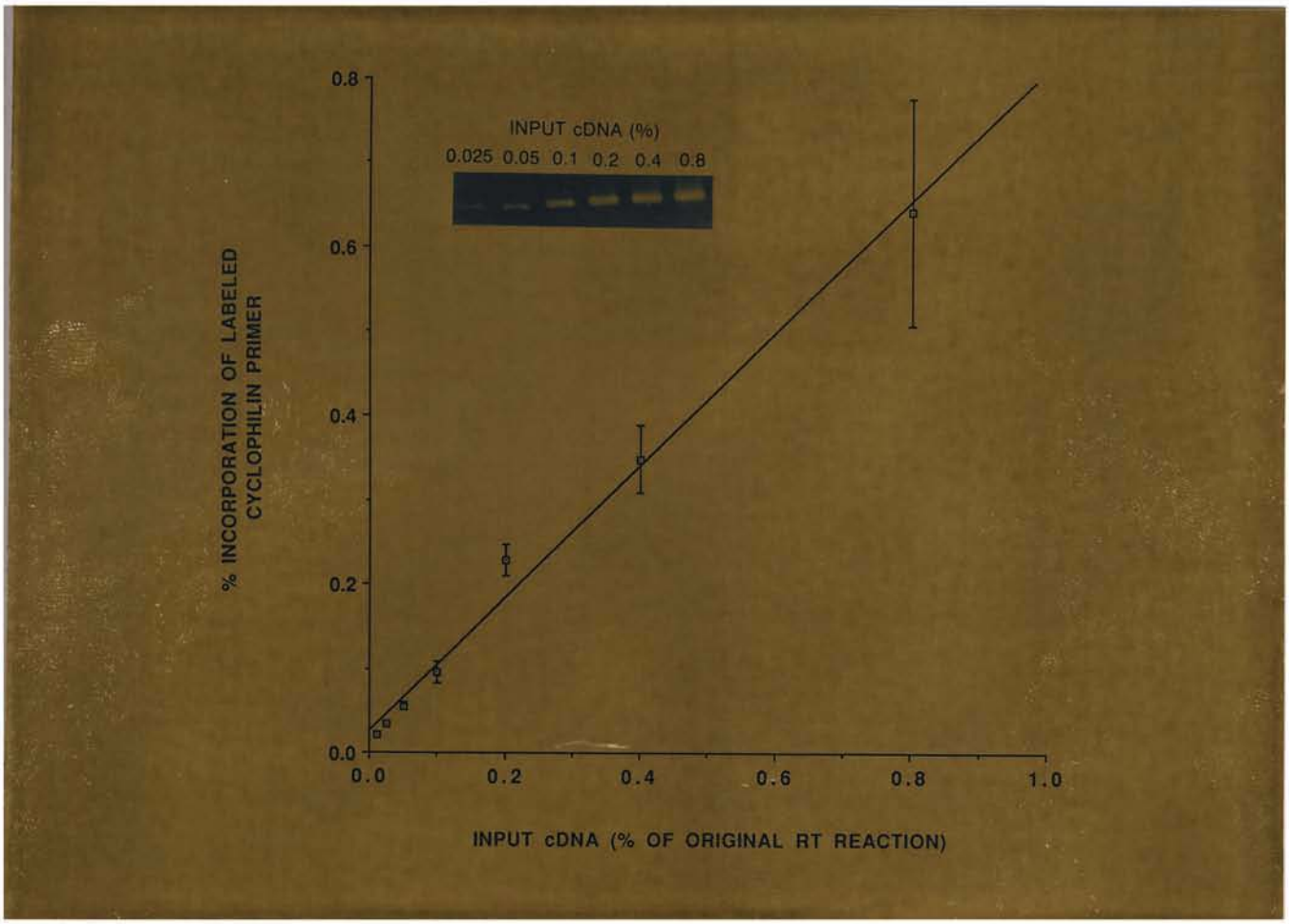
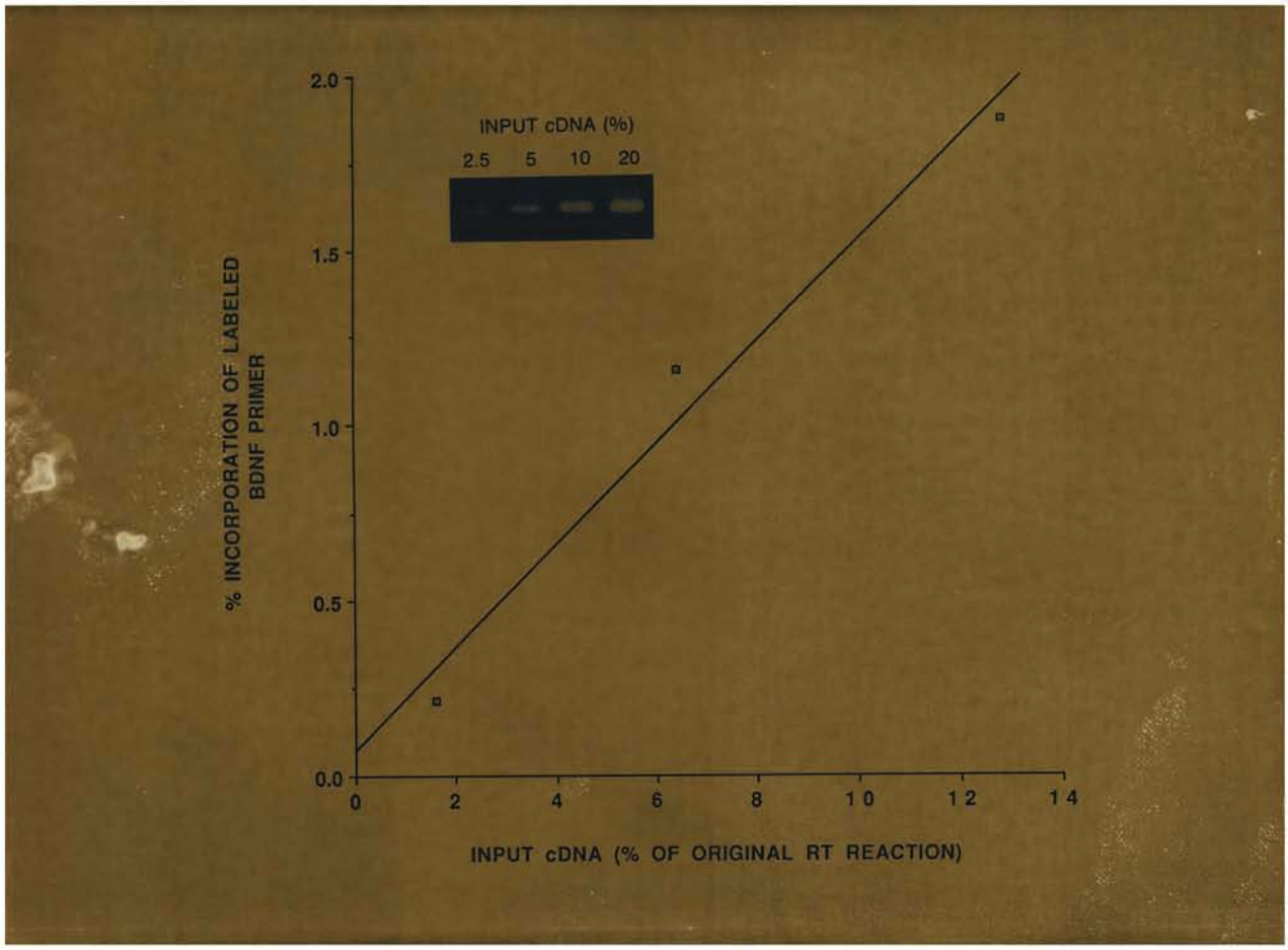

INPUT cDNA (%)
0.025 0.05 0.1 0.2 0.4 0.8
% INCORPORATION OF LABELED CYCLOPHILIN PRIMER
0.0
0.2
0.4
0.6
0.8
0.0
0.2
0.4
0.6
0.8
1.0
INPUT cDNA (% OF ORIGINAL RT REACTION)

Figure 8. Determination of optimal cDNA dilutions for quantitation of BDNF alternate 5′ exons. Total RNA from rat hippocampus was reverse transcribed using primer RT2 (Table 1). Serial dilutions of the resulting cDNA were subjected to QPCR using a primer set designed to amplify BDNF exon 1c (P5, P8, Table 1). Separation and quantitation of PCR products was carried out as described in the legend of Figure 7. Findings are taken from a single representative experiment and are comparable to similar analyses carried out using RNA from substantia nigra, hippocampus and cerebellum. Data points represent means ± S.E.M. (n=3). INSET shows the results of an identical experiment performed separately using unlabeled antisense primer.


INPUT cDNA (%)
2.5 5 10 20
% INCORPORATION OF LABELED BDNF PRIMER
2.0
1.5
1.0
0.5
0.0
0 2 4 6 8 10 12 14
INPUT cDNA (% OF ORIGINAL RT REACTION)

IV-3 Differential Expression of BDNF 5′ Exons in the Rat Brain

The relative expression of each BDNF 5′ exon in substantia nigra, striatum, hippocampus and cerebellum was measured by semi-quantitative PCR. The results confirmed that all BDNF 5' exons are found in each of the brain regions examined and that expression is always highest in the hippocampus, intermediate in the substantia nigra and cerebellum, and lowest in the striatum (Figure 9). However, the magnitude of these differences in expression varied for each 5′ exon. For example, the abundance of exon 1a transcripts in the cerebellum was 10% of that found in the hippocampus, whereas the abundance of exon 1c was 75% of that found in the hippocampus. In the substantia nigra, transcript levels ranged from 16% for exon 1a to 76% for exon 1b, relative to levels measured in the hippocampus. In the striatum, transcripts containing exons 1b and 1d were close to the threshold of detection in our standard 30 cycle QPCR assay. To estimate the concentrations of these very rare transcripts, all striatal cDNA samples were amplified again using increased PCR cycle numbers (Figure 10). Increasing cycle number from 30 to 35 produced large increases in the concentrations of PCR products arising from exons 1a, 1c and 1e cDNAs. However, these increases were not sustained when PCR cycles were further increased from 35 to 40, presumably due to depletion of reaction constituents or end product inhibition. Exon 1b transcripts were barely detectable after 35 cycles and exon 1d transcripts remained undetectable, even after 40 cycles. Based on the results obtained with 35 and 40 cycles of PCR, it appears that the expression of BDNF 5′ exons in the striatum follows, in diminishing order of concentration, 1c = 1e > 1a > 1b >> 1d.

Figure 9. Semi-quantitative PCR of BDNF alternate 5′ exon-containing transcripts in various brain regions. Optimal dilutions of BDNF or cyclophilin cDNAs were subjected to PCR using conditions designed to amplify either BDNF transcripts or cyclophilin transcripts using primers identified in Table 1. Separation and quantitation of PCR products was carried out as described in the legend of Figure 7. Results shown are ratios of BDNF alternate 5′ exon expression to that of cyclophilin in the same RNA sample. The expression of exons 1b and 1d was below reliable detection limits at 30 cycles of PCR. Data shown are means ± S.E.M (n = 4-6).

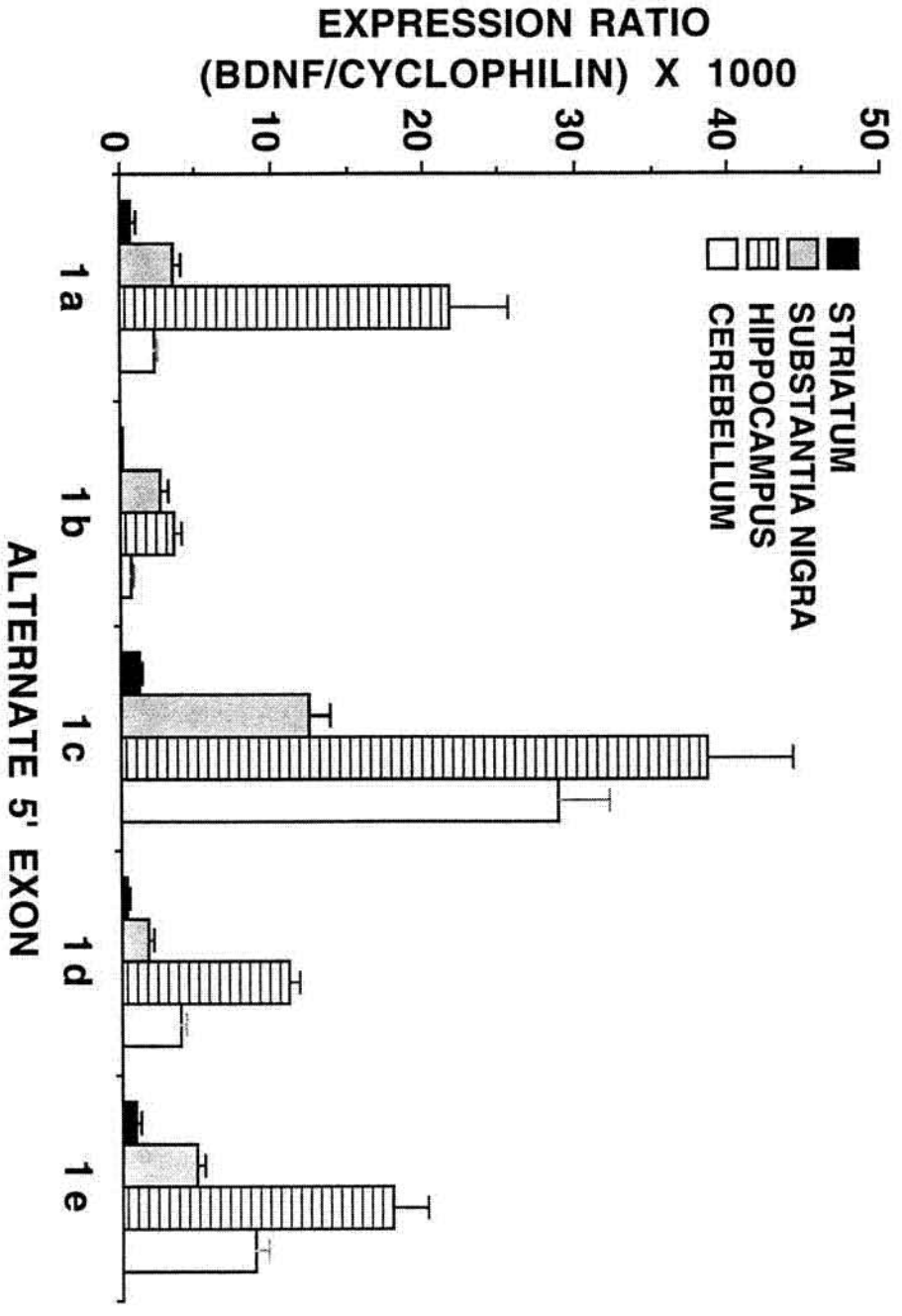
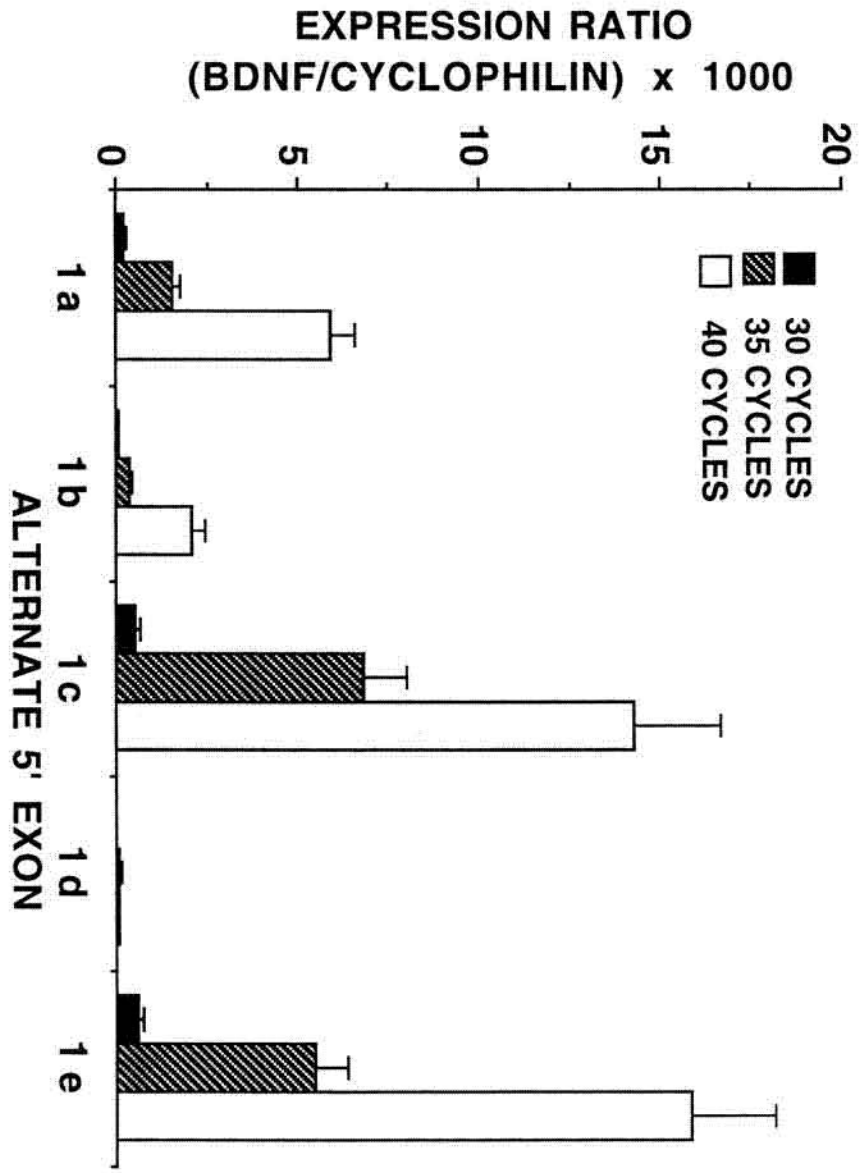

EXPRESSION RATIO
(BDNF/CYCLOPHILIN) X 1000
0
10
20
30
40
50
STRIATUM
SUBSTANTIA NIGRA
HIPPOCAMPUS
CEREBELLUM
1a
1b
1c
1d
1e
ALTERNATE 5' EXON

Figure 10. Semi-quantitative PCR of BDNF alternate 5′ exon-containing transcripts in the striatum using increasing PCR cycle numbers. Aliquots of PCR reactions were removed from each tube after 30, 35 and 40 cycles, the products were separated in agarose gels and the results were quantified as described in the legend of Figure 7. Note that exon 1d was undetectable even after 40 cycles of PCR. Data shown are means ± S.E.M. (n = 4-6).


EXPRESSION RATIO
(BDNF/CYCLOPHILIN) x 1000
0
5
10
15
20
30 CYCLES
35 CYCLES
40 CYCLES
1a
1b
1c
1d
1e
ALTERNATE 5' EXON

IV-4 Effects of 6-hydroxydopamine Lesions on BDNF mRNA Expression in the Rat Striatum

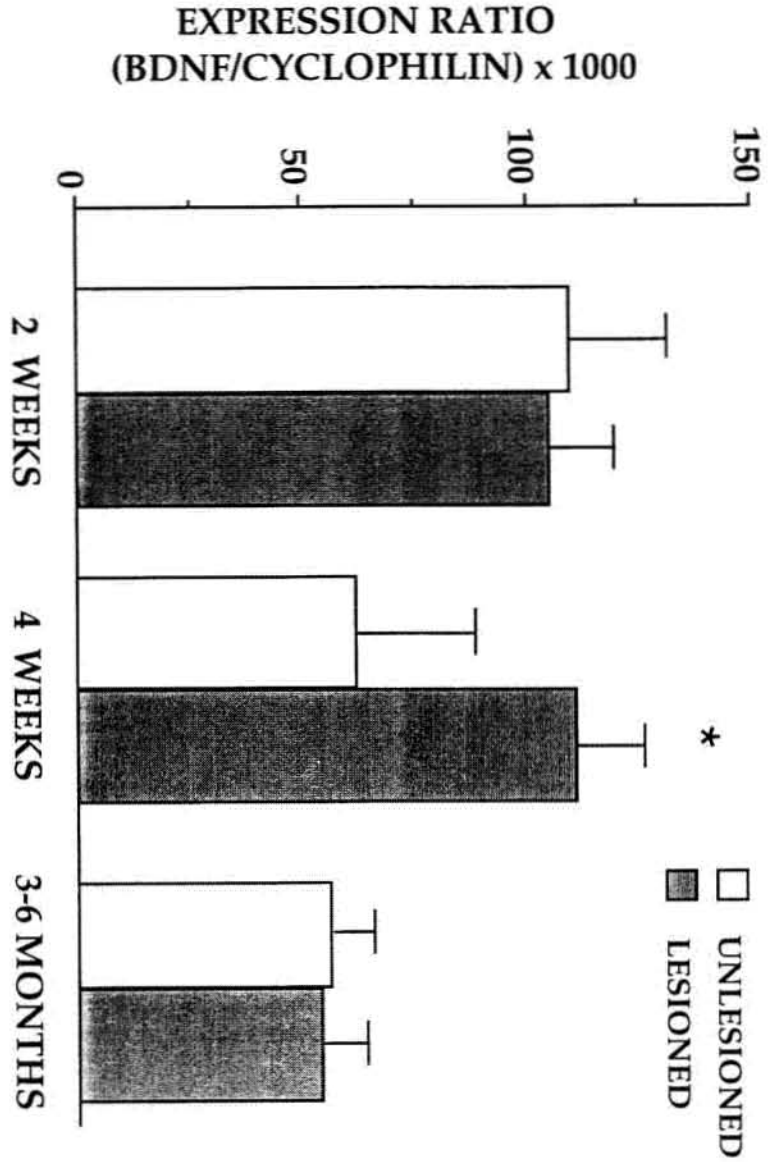
Three sets of rats with confirmed unilateral nigrostriatal lesions were sacrificed following 2 week, 4 week or 3-6 month survival times. Striata from both 6-OHDA lesioned and unlesioned sides of brains were dissected on ice and total RNA was extracted. Semi-quantitative measurements of BDNF coding exon transcript levels indicated significant effects of 6-OHDA lesions at the 4 week survival time only (Figure 11). However, it should be noted that there were no significant differences between cpm values obtained in the BDNF RT-PCR assays conducted on 6-OHDA treated versus control striata at this survival time. Instead, it was the cyclophilin values that were significantly decreased in the treated striata, which led to increased BDNF/cyclophilin ratios. The observed decrease in cyclophilin mRNA levels after 6-OHDA treatment was not seen at any other survival time, or in any of the other RT-PCR experiments conducted.

IV-5 Effects of Elevated Intracellular Calcium, cAMP or PKC Activity on BDNF and GDNF mRNA Expression in Rat C6 Glioma Cells

IV-5.a. General Characteristics of RT-PCR Assays

Semi-quantitative PCR assays were employed to measure levels of BDNF transcripts, as well as levels of more robustly expressed GDNF and cyclophilin transcripts in extracts of C6 glioma cells. The nucleotide sequences of all of the PCR products measured in these extracts were verified by DNA sequencing and/or restriction enzyme analysis (data not shown). As expected, the sequences of BDNF

Figure 11. Results of 6-OHDA lesion studies. Total RNA extracted from striata on lesioned and unlesioned sides of rat brains was subjected to semi-quantitative RT-PCR using the BDNF coding exon primer set (Table 1). Survival times for the three groups of rats studied were 2 weeks (n=5), 4 weeks (n=4) and 3-6 months (n=10) as indicted in the Figure. BDNF/cyclophilin expression ratios were calculated as described in section III-8.a, and the statistical signficance level was $*p < 0.05$, comparing lesioned to unlesioned conditions.


EXPRESSION RATIO
(BDNF/CYCLOPHILIN) x 1000
0
50
100
150
2 WEEKS
4 WEEKS
3-6 MONTHS
*
UNLESIONED
LESIONED

PCR products derived from C6 cell RNA were identical to those described for products derived from rat brain RNA. GDNF RT-PCR produced 2 bands, 364 bp and 286 bp, which correspond to the published sequence and a recently described splice variant (Schaar et al. 1994). Although both of these products were consistently visualized, excised and subjected to scintillation counting, only the longer product was included in cDNA concentration-percent incorporation curves and subsequent RT-PCR assays, since the shorter transcript was much less abundant.

Concentration-percent incorporation curves were generated from C6 cell cDNA and used to establish the linear ranges of the PCR assays employed. As shown in Figures 12, 13 and 14, the linear range of input cDNA was 3.125 to 50 percent, 1.25 to 20 percent and 0.039 to 0.625 percent of standard RT reactions for measurement of BDNF coding exon, GDNF and cyclophilin transcripts, respectively. Semi-quantitative PCR assays designed to measure alternate BDNF exon-containing transcripts in C6 cells were adjusted in a similar manner (data not shown). In subsequent assays, GDNF and all BDNF transcript levels were normalized to cyclophilin transcript levels in each sample. GDNF mRNA levels in C6 cells appeared to be higher than BDNF mRNA levels, requiring only 5% of a standard RT reaction to measure basal expression with 30 PCR cycles, compared with 12.5% for BDNF. Among the five alternate BDNF first exons, transcripts containing exon 1e were most abundant, requiring 30 cycles of PCR, followed by exons 1b and 1d, requiring 35 cycles. The expression levels of the other two known alternate BDNF exons, 1a and 1c, were very low in C6 cells, requiring 40 cyles of PCR to visualize a band. However, levels of background, nonspecific bands were unacceptable so these exons were excluded from further examination.

Figure 12. Concentration-percent incorporation curve for semi-quantitative BDNF RT-PCR assays conducted on C6 glioma cells. Data points represent means ± S.E.M. (n=3) and the dashed regression lines were drawn through the linear portion of the curve ($R^2 > 0.97$). The BDNF curve was obtained by pooling cDNA made from C6 cell total RNA primed with RT2 (Table 1), performing serial dilutions in RT buffer and subjecting aliquots of the diluted cDNA to BDNF RT-PCR (30 cycles) using P1 and P2 primers. Radioactive counts from the excised bands were converted to percent incorporation by dividing them by the total counts added per PCR reaction tube. The inset is a photograph of PCR products resulting from a selected dilution series used to construct the graph.

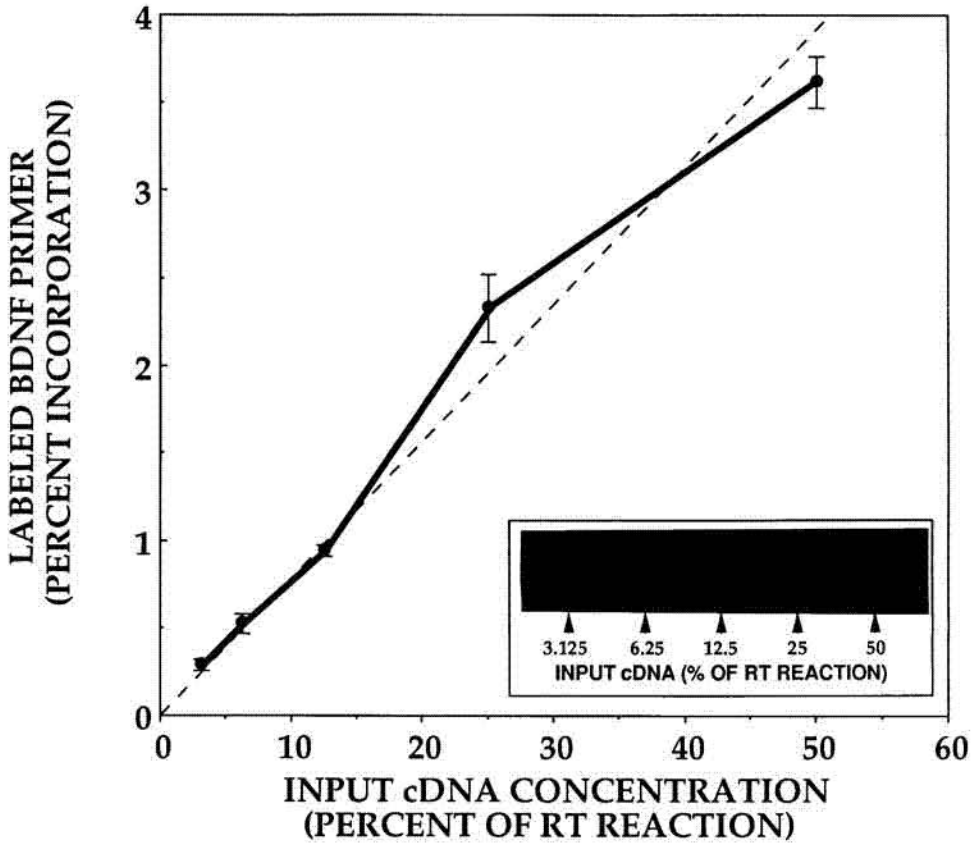

LABELED BDNF PRIMER
(PERCENT INCORPORATION)
0
1
2
3
4
0
10
20
30
40
50
60
INPUT cDNA CONCENTRATION
(PERCENT OF RT REACTION)
3.125
6.25
12.5
25
50
INPUT cDNA (% OF RT REACTION)

Figure 13. Concentration-percent incorporation curve for semi-quantitative GDNF RT-PCR assays conducted on C6 glioma cells. Data points represent means ± S.E.M. (n=3) and the dashed regression lines were drawn through the linear portion of the curve ($R^2 > 0.97$). The GDNF curve was obtained by pooling cDNA made from C6 cell total RNA primed with RT3 (Table 1), performing serial dilutions in RT buffer and subjecting aliquots of the diluted cDNA to GDNF RT-PCR (30 cycles) using P9 and P10 primers. Radioactive counts from the excised bands were converted to percent incorporation by dividing them by the total counts added per PCR reaction tube. The lower percent incorporation for GDNF PCR products compared with BDNF and cyclophilin (Figures 12 and 14) is probably due to lower primer binding efficiency commensurate with the elevated annealing temperature required (64 oC) in this assay. The inset is a photograph of PCR products resulting from a selected dilution series used to construct the graph.

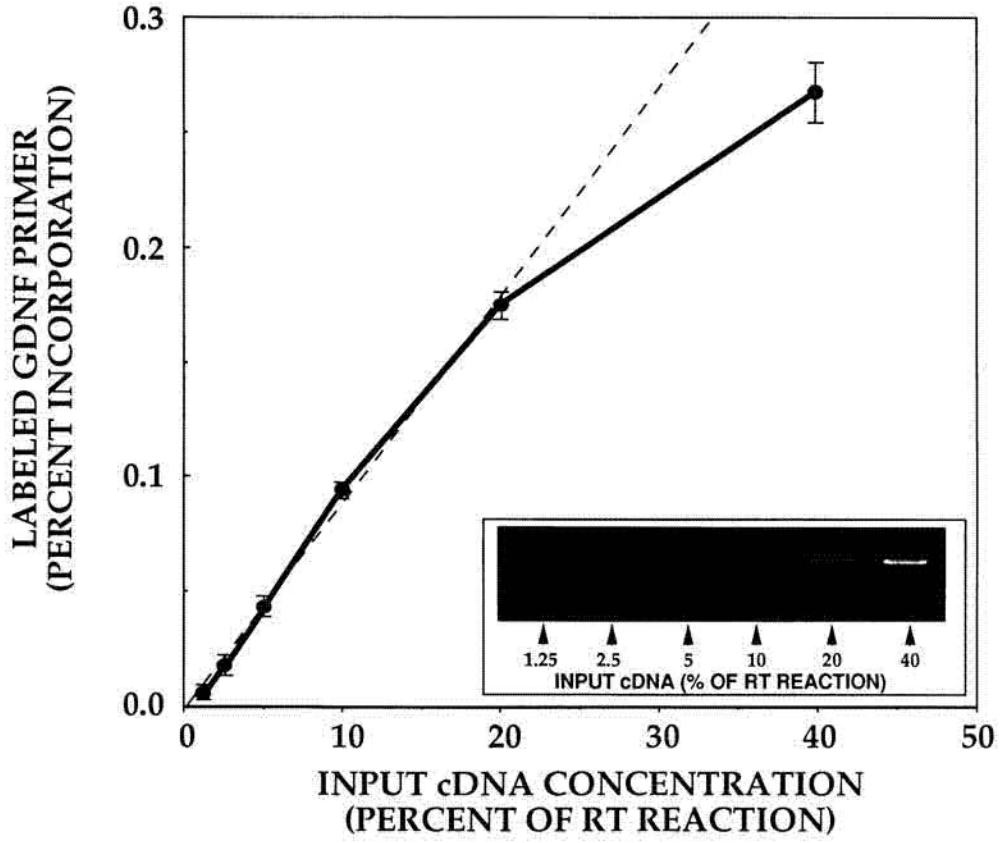
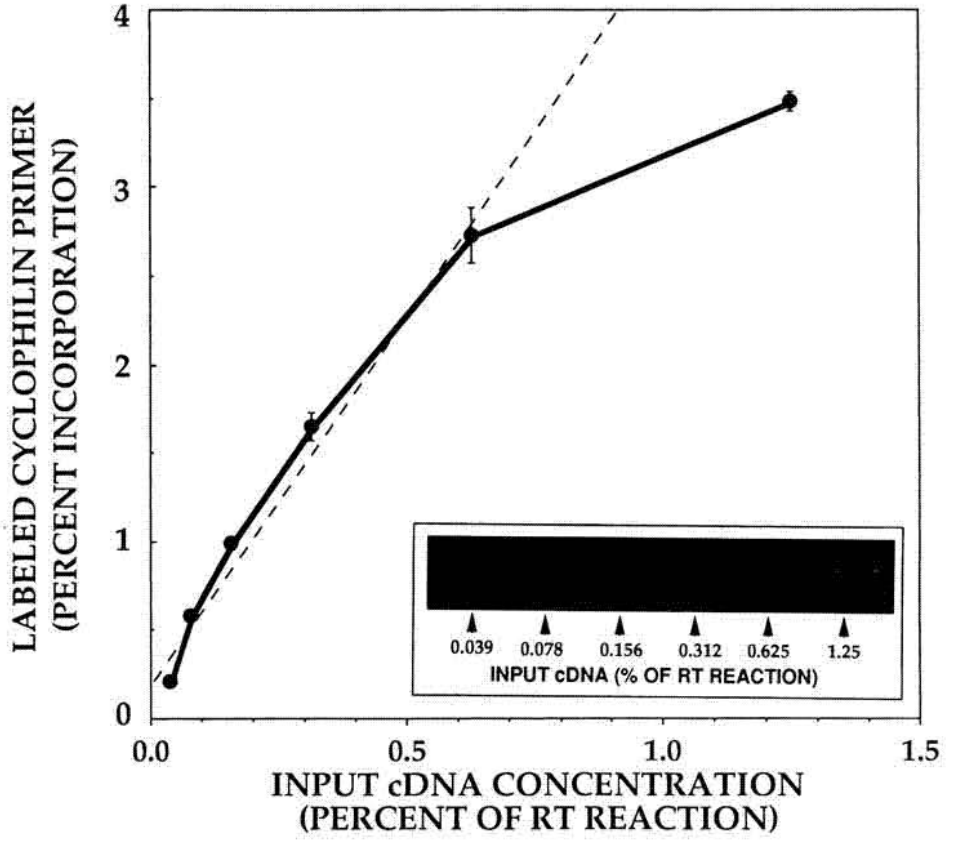

LABELED GDNF PRIMER
(PERCENT INCORPORATION)
0.3
0.2
0.1
0.0
0
10
20
30
40
50
INPUT cDNA CONCENTRATION
(PERCENT OF RT REACTION)
1.25
2.5
5
10
20
40
INPUT cDNA (% OF RT REACTION)

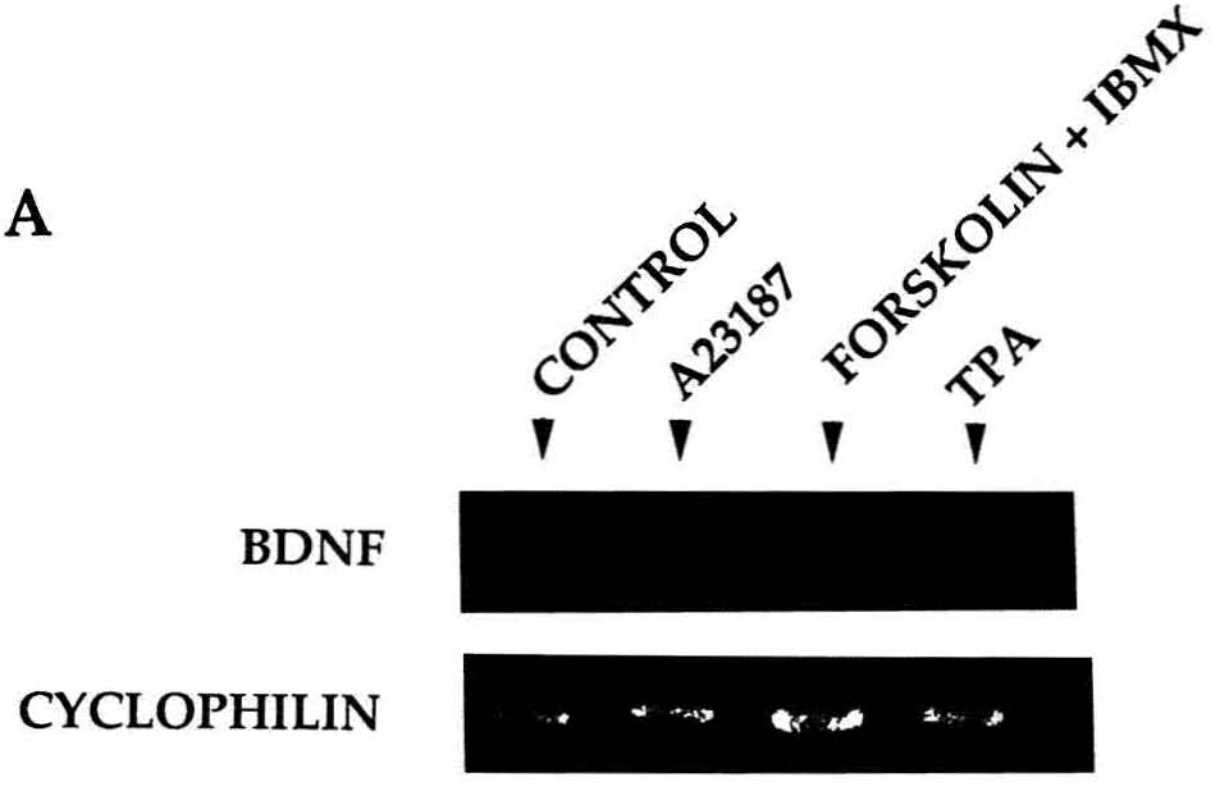
Figure 14. Concentration-percent incorporation curve for semi-quantitative cyclophilin RT-PCR assays conducted on C6 glioma cells. Data points represent means ± S.E.M. (n=3) and the dashed regression lines were drawn through the linear portion of the curve ($R^2 > 0.97$). The cyclophilin curve was obtained by pooling cDNA made from C6 cell total RNA primed with RT4 (Table 1), performing serial dilutions in RT buffer and subjecting aliquots of the diluted cDNA to GDNF RT-PCR (30 cycles) using P11 and P12 primers. Radioactive counts from the excised bands were converted to percent incorporation by dividing them by the total counts added per PCR reaction tube. The inset is a photograph of PCR products resulting from a selected dilution series used to construct the graph.


LABELED CYCLOPHILIN PRIMER
(PERCENT INCORPORATION)
4
3
2
1
0
0.0
0.5
1.0
1.5
INPUT cDNA CONCENTRATION
(PERCENT OF RT REACTION)
0.039
0.078
0.156
0.312
0.625
1.25
INPUT cDNA (% OF RT REACTION)

IV-5.b. Treatment Effects on BDNF mRNA Expression

RNA samples from C6 cells exposed to A23187, forskolin + IBMX, TPA or vehicle, as described in section III-6, were subjected to semi-quantitative RT-PCR using BDNF exon primers (RT2, P1 and P2). In a representative experiment, a 6.4-fold increase ($p < 0.0001$) and a 2.4-fold increase ($p < 0.01$) in BDNF coding exon expression was observed in A23187 and forskolin + IBMX treated cells, respectively (Figure 15A, 15B); whereas TPA treatment was ineffective. Each of the three alternate 5′ exon-containing transcripts analyzed was significantly elevated after A23187 treatment ($p < 0.001$, Figure 15 C-15E). However, only exon 1e-containing transcripts were significantly elevated after forskolin + IBMX treatment ($p < 0.01$, Figure 15E) and none of the alternate transcripts were affected by TPA treatment. Actinomycin D, an inhibitor of RNA polymerase, had no effect on basal BDNF coding exon expression but nearly prevented the A23187-induced response ($p < 0.01$, Figure 16A, 16B). As seen for the BDNF coding exon, exon 1e-containing transcripts were also significantly reduced ($p < 0.01$), but not abolished, by actinomycin D in A23187 treated cells (Figure 16E). A23187-induced increases in BDNF transcripts containing exons 1b or 1d were totally abolished by coincubation with actinomycin D (Figure 16C, 16D).

Figure 15. Effects of second messenger-inducing compounds on BDNF mRNA levels. Based on the concentration-percent incorporation curves, the following amounts of cDNA were included in BDNF semi-quantitative PCR assays: 12.5% and 25% of a standard RT reaction for the BDNF coding exon and alternate exon 1e, respectively, subjected to 30 cycles of PCR; 25% for alternate exons 1b and 1d in 35 cycle PCR assays. Cyclophilin mRNA concentrations used to normalize the BDNF data were determined as described in Materials and Methods. A) Representative experiment (n=3) showing the effects of A23187, forskolin + IBMX or TPA on BDNF coding exon expression compared to vehicle-treated controls. Cyclophilin products resulting from the same RT reactions are also shown. B-E) Summary data assembled from the experiment shown in (A) are means ± S.E.M (n = 3) for the coding exon (B); exon 1b (C); exon 1d (D); and exon 1e (E), ***$p < 0.0001$, **$p < 0.001$, and *$p < 0.01$ compared with controls. All treatments were performed at least three times (n = 3-6) with similar results.


A
CONTROL
A23187
FORSKOLIN + IBMX
TPA
BDNF
CYCLOPHILIN

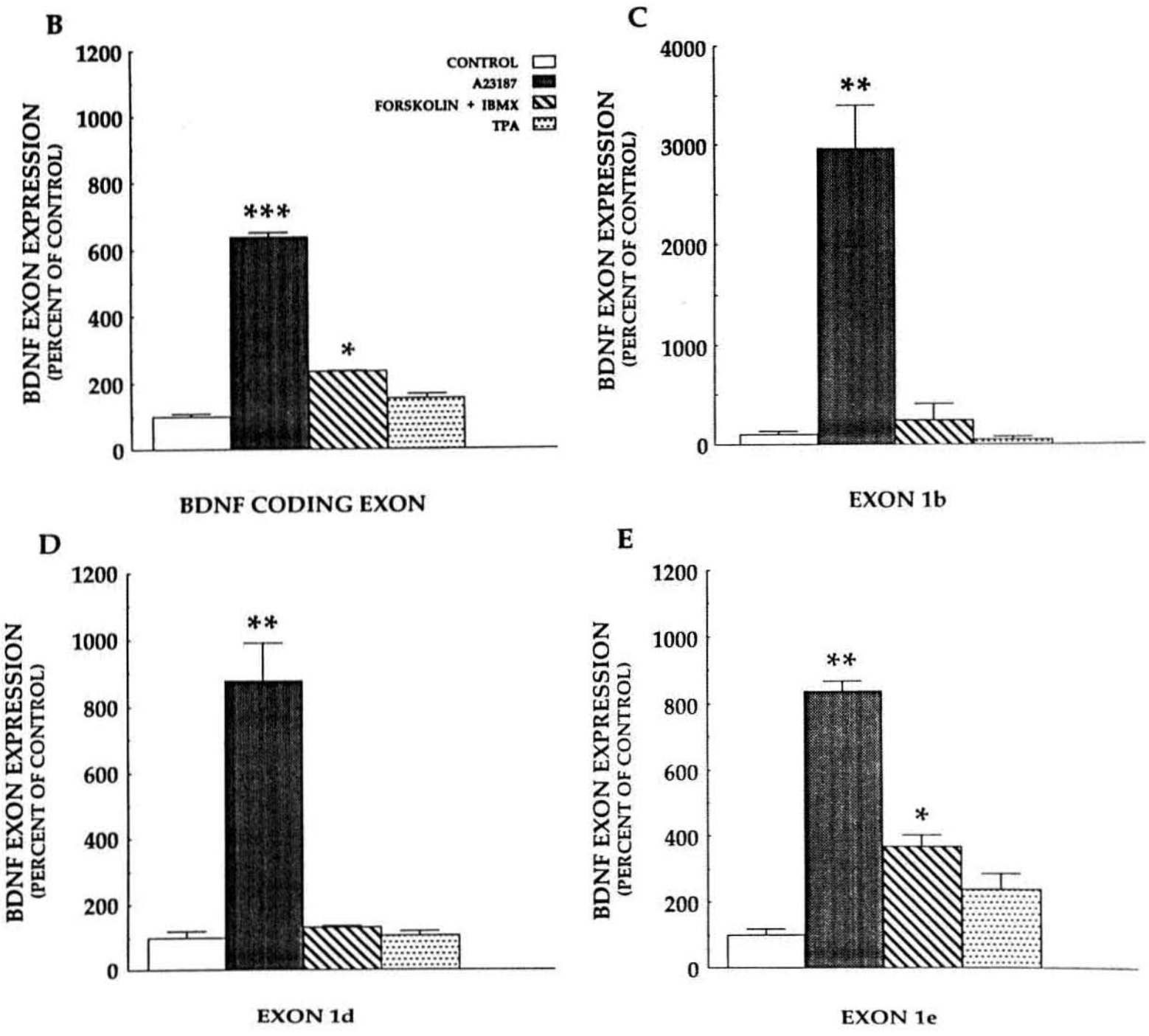
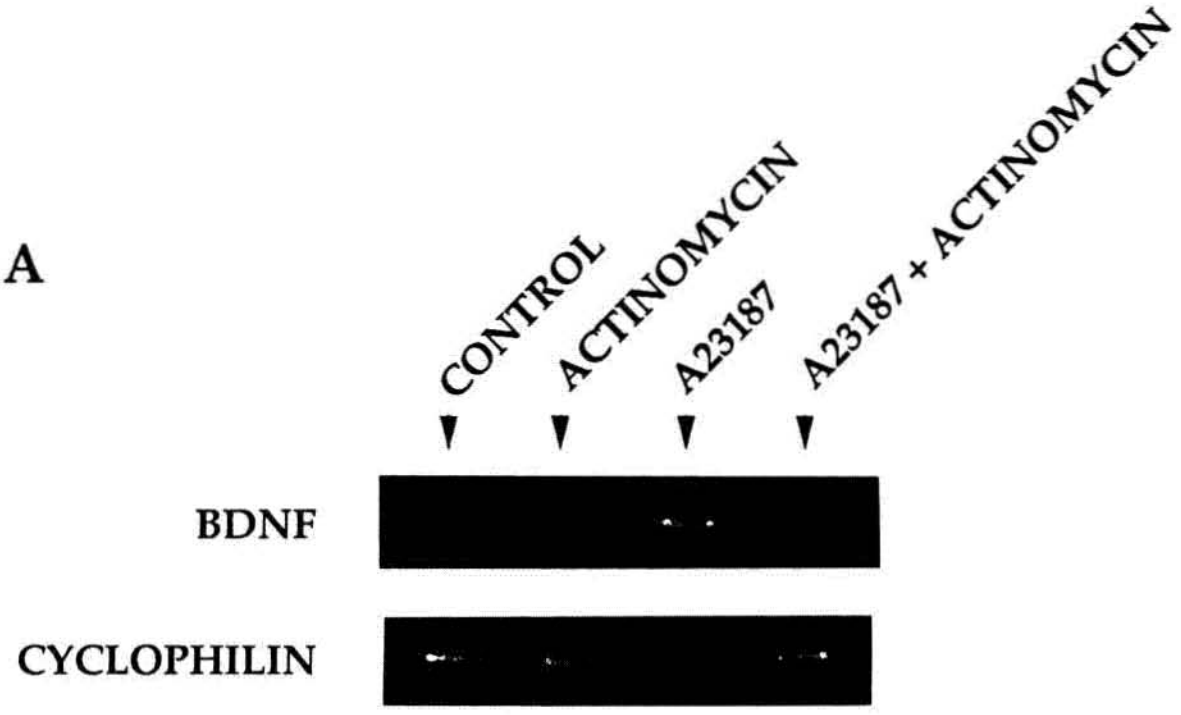
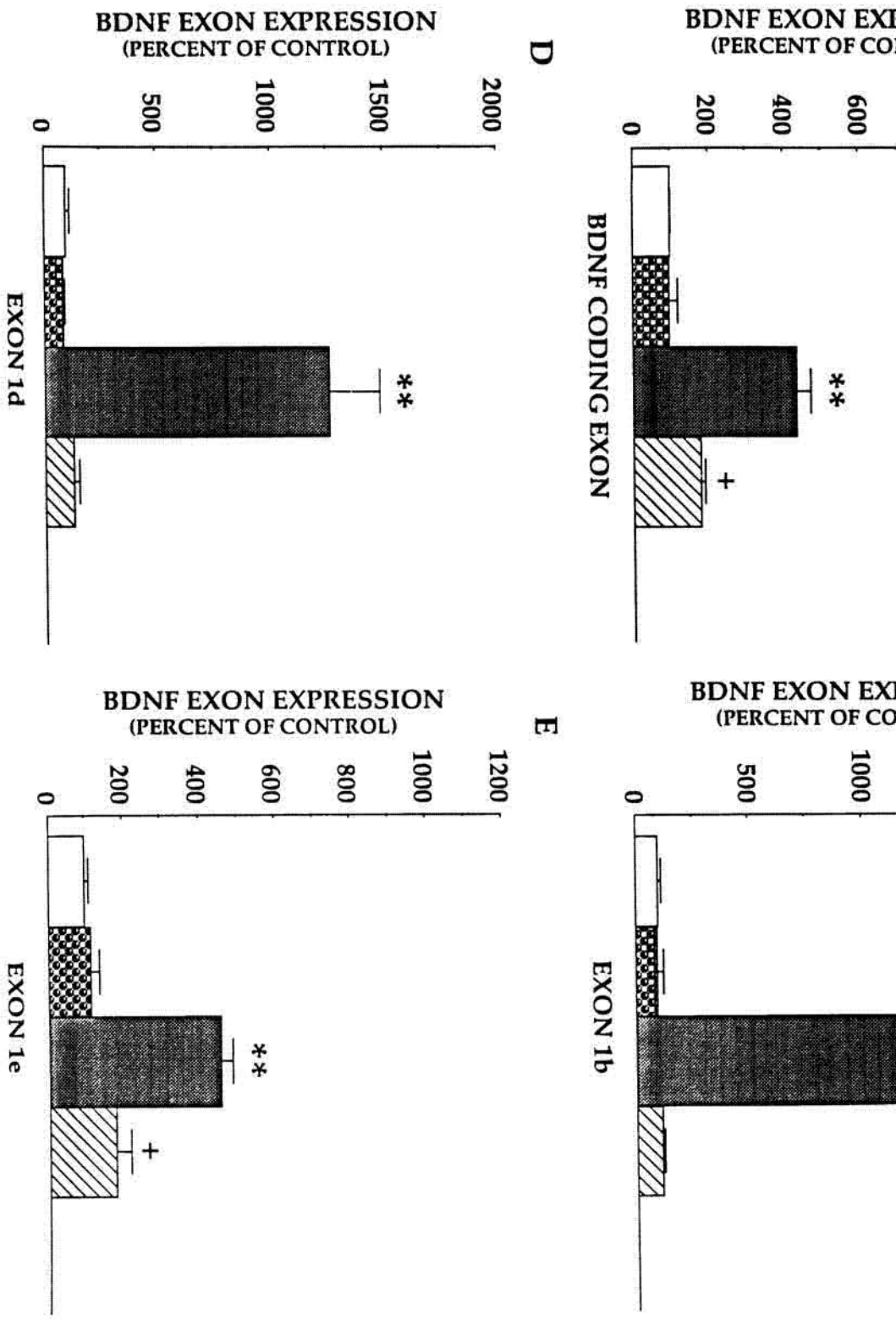

B
BDNF EXON EXPRESSION
(PERCENT OF CONTROL)
1200
1000
800
600
400
200
0
CONTROL
A23187
FORSKOLIN + IBMX
TPA
***
*
BDNF CODING EXON
C
BDNF EXON EXPRESSION
(PERCENT OF CONTROL)
4000
3000
2000
1000
0
**
EXON 1b
D
BDNF EXON EXPRESSION
(PERCENT OF CONTROL)
1200
1000
800
600
400
200
0
**
EXON 1d
E
BDNF EXON EXPRESSION
(PERCENT OF CONTROL)
1200
1000
800
600
400
200
0
**
*
EXON 1e

Figure 16. Effects of actinomycin D on A23187-induced BDNF expression. A) shows a photograph of PCR products observed in a typical experiment measuring levels of the BDNF coding exon and (B-E) depict summary data for the coding exon (B); exon 1b (C); exon 1d (D); and exon 1e (E) from the same experiment (n= 4-6), $^{**}p < 0.001$ compared to controls; and $^{+}p < 0.01$, compared with A23187 treatment. The dose of actinomycin D was 10 μg/ml and the control solution contained 0.01% DMSO. All treatments were performed at least three times (n = 3-6) with similar results.

A

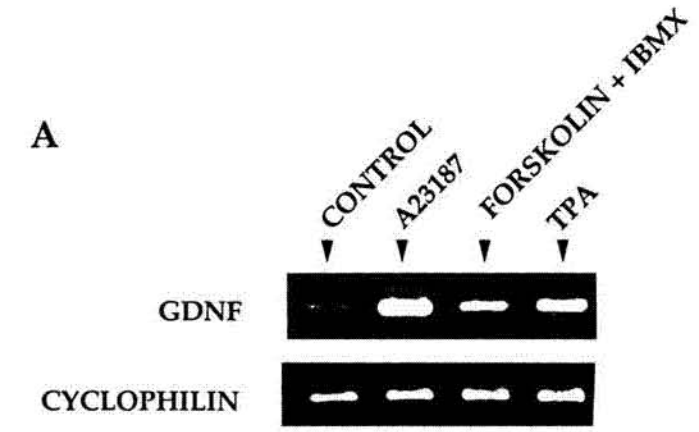

CONTROL
ACTINOMYCIN
A23187
A23187 + ACTINOMYCIN
BDNF
CYCLOPHILIN


B
BDNF EXON EXPRESSION
(PERCENT OF CONTROL)
0
200
400
600
800
1000
1200
BDNF CODING EXON
**
+
CONTROL
ACTINOMYCIN
A23187
A23187 + ACTINOMYCIN
C
BDNF EXON EXPRESSION
(PERCENT OF CONTROL)
0
500
1000
1500
2000
EXON 1b
**
D
BDNF EXON EXPRESSION
(PERCENT OF CONTROL)
0
500
1000
1500
2000
EXON 1d
**
E
BDNF EXON EXPRESSION
(PERCENT OF CONTROL)
0
200
400
600
800
1000
1200
EXON 1e
**
+

IV-5.c. Treatment Effects on GDNF mRNA Expression

C6 cells were treated for 4 hours with A23187, forskolin + IBMX, TPA or vehicle, total RNA was extracted and subjected to semi-quantitative RT-PCR using GDNF primers (RT3, P9 and P10). Treatment with the calcium ionophore resulted in a 7-fold increase in GDNF transcript levels ($p < 0.001$, Figure 17A, 17B). By comparison, forskolin + IBMX and TPA treatments were less effective. Forskolin + IBMX had no effect on GDNF expression, while TPA treatment produced a 3.3-fold elevation ($p < 0.05$, Figure 17B). In all experiments, treatment-induced changes in levels of the 364 bp GDNF QPCR product were mirrored by changes in levels of the 286 bp product (data not shown). To determine if the robust elevation in GDNF mRNA levels seen after A23187 treatment was due to control of transcription or post-transcriptional processing, an RNA polymerase inhibitor was used. In the presence of actinomycin D, basal GDNF expression was abolished and A23187-induced expression was significantly reduced ($p < 0.01$), but remained elevated at 243% of vehicle-treated control values (Figure 18). Each experiment was performed at least three times with similar results.

Figure 17. Effects of second messenger-inducing compounds on GDNF mRNA levels. A) Representative experiment showing the effects of A23187, forskolin + IBMX or TPA on intensities of GDNF semi-quantitative PCR products compared to vehicle-treated controls. Cyclophilin products resulting from the same RT reactions are also shown. B) Summary data assembled from the experiment shown in (A) are means ± S.E.M (n = 3), $^{**}p < 0.001$; $^{\#}p < 0.05$ compared with vehicle-treated controls. All treatments were performed at least twice (n = 3-6) with similar results. The S.E.M. for the A23187 treatment condition is too small be be seen on this scale.


A
CONTROL
A23187
FORSKOLIN + IBMX
TPA
GDNF
CYCLOPHILIN

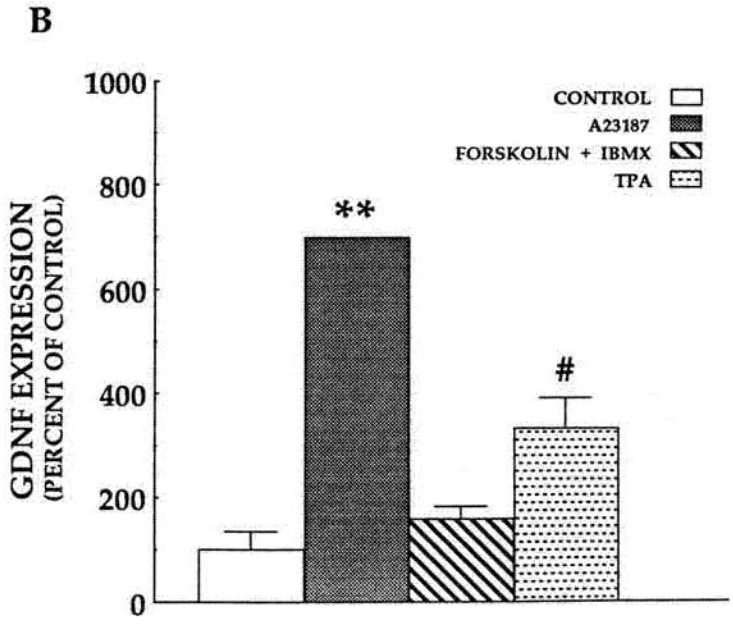
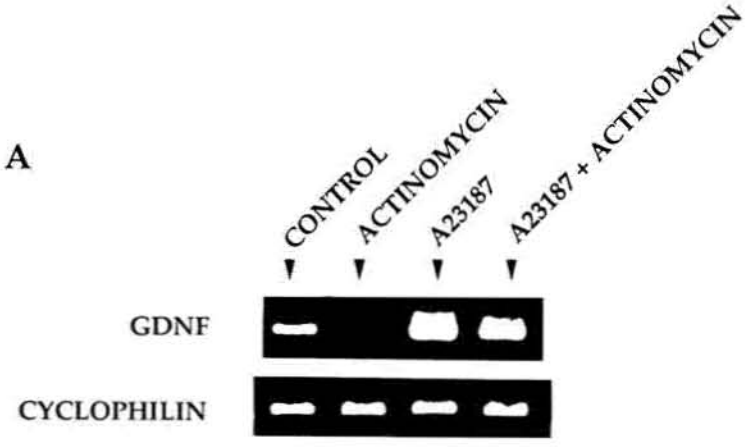
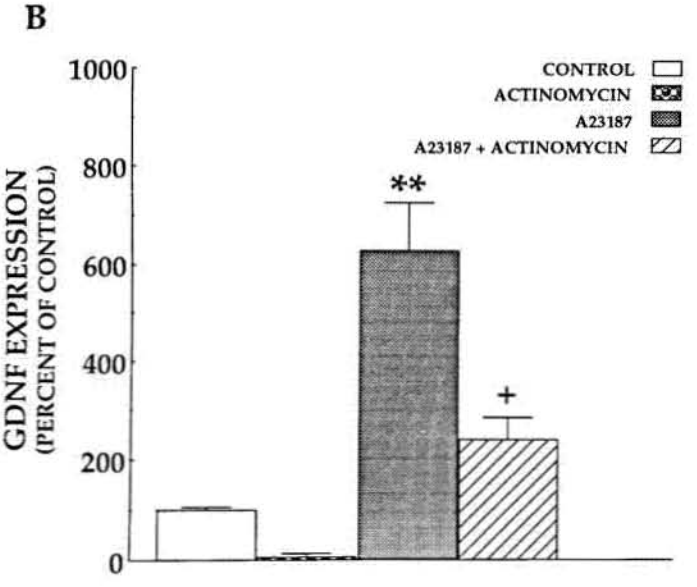

B
GDNF EXPRESSION
(PERCENT OF CONTROL)
1000
800
600
400
200
0
CONTROL
A23187
FORSKOLIN + IBMX
TPA
**
#

Figure 18. Effects of actinomycin D on A23187-induced GDNF expression. A) shows a photograph of PCR products observed in a typical experiment and (B) depicts summary data from the same experiment (n= 4-6), mean ± S.E.M (n = 3), $^{**}p < 0.001$ compared to controls; and $^{+}p < 0.01$ compared to A23187 treatment. The dose of actinomycin D was 10 μg/ml and the control solution contained 0.01% DMSO. All treatments were performed at least three times (n = 3-6) with similar results.

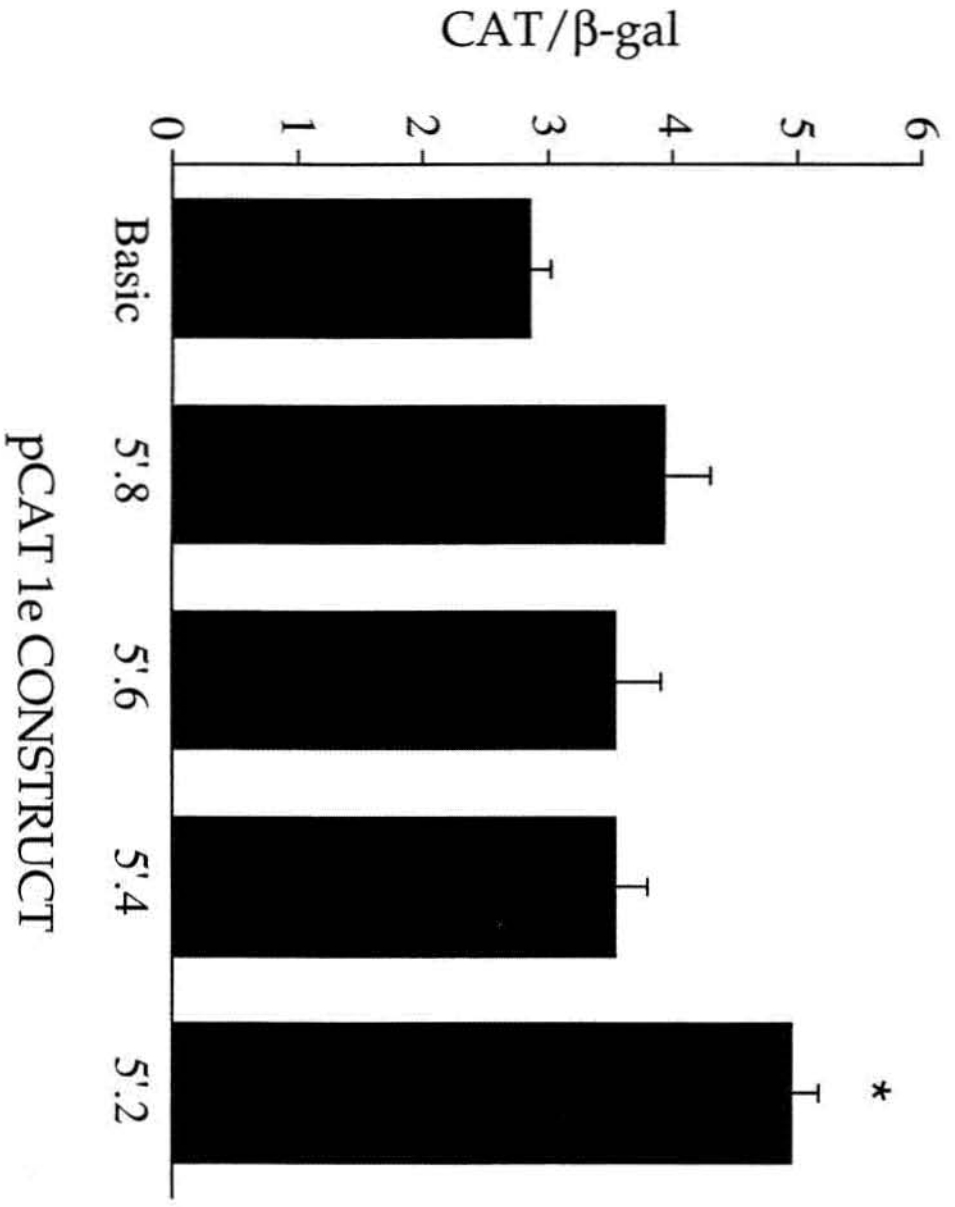

A
CONTROL
ACTINOMYCIN
A23187
A23187 + ACTINOMYCIN
GDNF
CYCLOPHILIN


B
GDNF EXPRESSION
(PERCENT OF CONTROL)
1000
800
600
400
200
0
CONTROL
ACTINOMYCIN
A23187
A23187 + ACTINOMYCIN
**
+

IV-5.d.Characterization of BDNF Exon 1e Promoter Mechanics

Since exon 1e-containing transcripts are the most prevalent BDNF transcripts in C6 cells, experiments were conducted to localize and assess the strength of putative *cis*-acting elements in the 1e promoter. Transfection of CAT reporter vectors containing sequentially shortened fragments of the 1e promoter region (Figure 4) into C6 cells, along with a β-gal producing vector used to normalize DNA uptake, resulted in differential CAT expression (Figure 19). Only pCAT1e-5'.2 produced statistically significant elevations in CAT enzyme levels ($p < .0001$), ranging from 1.4-fold to 2.2-fold across experiments, as compared to results obtained by transfection of the parent vector, pCAT-Basic.

Figure 19. CAT activity conferred by sequentially shortened segments of the BDNF exon 1e promoter. Ten centimeter dishes of C6 cells were transfected with 10 μg of pCAT Basic or a pCAT1e-5′ deletion construct along with 5 μg of a β-galactosidase reporter construct as described in section III-7.a. Approximately 72 hours later, the cell were lysed and lysates were subjected to CAT and β-gal assays as described in section III-7.c. The graph depicts the resulting CAT/β-gal ratios averaged over 3 experiments (mean ± S.E.M, n = 3 or 4 per experiment). The asterisk signifies $p < 0.0001$).


CAT/β-gal
0
1
2
3
4
5
6
Basic
5'.8
5'.6
5'.4
5'.2
*
pCAT 1e CONSTRUCT

Chapter V Discussion

V-1 *Overview*

Mounting evidence indicates that BDNF and GDNF have potent trophic effects on degenerating neurons in several areas of the CNS. This raises the possibility that these factors might be useful for the treatment of neurodegenerative disorders (Eide et al. 1993; Lindsay et al. 1993; Acheson and Lindsay, 1994; Tuszynski and Gage, 1994; Lindsay, 1995). For example, the trophic and protective effects of BDNF and GDNF on dopaminergic cells, demonstrated both *in vitro* (Hyman et al. 1991; Spina et al. 1992; Knusel et al. 1991; Oppenheim et al. 1995) and *in vivo* (Altar et al. 1992; Shults et al. 1994; Hoffer et al. 1994; Tomac et al. 1995a), render characterization of the molecular mechanisms responsible for regulating BDNF and GDNF gene transcription an important aspect of a multifaceted approach to the treatment of Parkinson's disease. Since, symptoms are not observed until more than 80% of the dopaminergic neurons are lost, protection of as few as 20% of these neurons could allow persons afflicted with this disease to live normal lives (Lindsay et al. 1994). One approach that could be used to increase the availability of neurotrophins to degenerating cells is to augment their endogenous production. As such, understanding the mechanisms responsible for modulating BDNF and GDNF expression is an important research goal.

This dissertation focused on intracellular mechanisms involved in the regulation of BDNF and GDNF mRNA expression. The work presented here and by other laboratories has confirmed the existence of the four BDNF 5′ exons discovered by Timmusk et al. (1993), and demonstrates that BDNF transcripts in the rat brain are comprised of splice variants containing these four exons as well as a novel fifth

exon, exon 1a, which was discovered during the course of this dissertation. Further experiments showed that BDNF mRNA expression in the rat brain is primarily due to differential activation of multiple promoters located adjacent to these exons. It was also shown that, based on tissue culture studies employing C6 glioma cells, changes in BDNF and GDNF mRNA levels are mainly due to changes in transcription of these genes, as opposed to post-transcriptional processing. These latter studies have also provided evidence that transcription of both of these genes is modulated by changes in intracellular calcium, with minor contributions by other prominent signal transduction pathways. The following sections will review and interpret cogent aspects of BDNF alternate exon and GDNF mRNA expression in rat brain and tissue culture.

V-2 BDNF and Parkinson's Disease

BDNF mRNA in the adult rat brain is found in highest concentrations in the hippocampus and cerebellum with lower concentrations present in other areas of the forebrain, midbrain and hindbrain (Maisonpierre et al. 1990; Hofer et al. 1990). BDNF mRNA levels in the substantia nigra are very low and levels in the striatum were undetectable by conventional Northern blot analysis (Maisonpierre et al. 1990). Although levels appear to be low in the regions surrounding the cell bodies and the terminal fields of the nigro-striatal dopaminergic neurons, this does not preclude a physiologic role for BDNF in the proper function of these neurons. As introduced earlier, a single oral dose of L-dopa led to increased concentrations of BDNF mRNA in the striatum (Okazawa et al. 1992), suggesting that the expression of BDNF is coordinated with the activity of the neurons it supports. Since degeneration of the dopaminergic neurons in Parkinson's disease leads to a decrease in dopamine

release in the striatum, perhaps some of the symptoms of Parkinson's disease might be attributed to a resulting decrease in BDNF expression in this region. If this is indeed the case, the development of methods to increase BDNF expression in the striatum will be an important therapeutic goal for the treatment of Parkinson's disease. Thus, although BDNF mRNA levels in the basal ganglia are low, the large accumulation of evidence supporting a role for BDNF in the nourishment of dopaminergic cells (section I-6.b) makes the investigation of the regulatory mechanisms responsible for BDNF gene expression an important research goal.

V-3 Alternate 5′ Exons in the BDNF Gene

V-3.a. BDNF Exon 1a

The results of the 5′ RACE and DNA sequencing procedures indicated that all four of the alternate 5′ exons reported by Timmusk et al. (1993), labeled 1b, 1c, 1d, and 1e in this dissertation, are expressed in the cerebellum. In addition, a previously unreported 5′ exon (exon 1a) is expressed in this brain region as well as in substantia nigra, striatum and hippocampus. Although the location of this exon, with respect to the other four exons, is still uncertain, there is evidence to support the prediction that it is located about 1 kb downstream of exon 1c (Figure 4, T. Timmusk, personal communication). Since exon 1a is contained in a rare BDNF transcript that was only observed in 1 out of 70 5′ RACE clones, confirmation that the exon 1a transcript is a legitimate BDNF mRNA species required additional procedures. RT-PCR experiments carried out on hippocampal RNA revealed a RACE product of the predicted size (Figure 5), which was verified by restriction mapping and Northern blot analysis (Figure 6). Thus, we conclude that the 1a transcript is an authentic

BDNF transcript that is very rare, which may account for the lack of its discovery by other investigators. Perhaps the promoter controlling expression of this transcript is normally repressed and becomes active only under specific circumstances, such as a particular insult in a particular cell type.

As mentioned in the Results, exon 1b was the only alternate BDNF exon reported to date that was not found in any of the 5′ RACE clones obtained in the present work. Based on the sequence of this exon, published originally by Timmusk et al. (1993) and later by Nakayama et al. (1994), an exon 1b primer (P4) was paired with a BDNF coding exon primer (P8) and a 1b exon PCR product was generated and confirmed by DNA sequencing (see Figure 3). It is possible that the failure to obtain this exon via 5′ RACE is due to the very low level of constituitive expression of this transcript in all tissues examined so far (Timmusk et al. 1993; Nakayama et al. 1994; this dissertation).

V-3.b. BDNF Exon 1c

Of the seventy RACE clones sequenced, the majority contained the exon 1c sequence shown in Figure 3, corresponding to a portion of one of the splice variants of this exon (exon II) reported by Timmusk et al. (1993). Out of a total of 15 exon 1c clones, 11 had identical but shorter sequences and 4 were splice variants, either extended at the 5′ end or containing additional segments within the sequence shown in Figure 3. This sequence complexity in exon 1c-containing transcripts investigated in the present work is in agreement with later reports of complex splicing patterns for this exon (Timmusk et al. 1993; Nakayama et al. 1994). Moreover, the pattern observed in the rat cerebellum is especially complex, containing a multitude of unique splice variants (M. Metsis, personal

communication). Whereas Timmusk et al. (1993) did not specify which of the 1c (exon II) splice variants were most prevalent, the sequence of this transcript given in their article was identical to one of the variants observed in the present work. Since these investigators reported a number of putative translation start sites located in the same region as the putative 5′ cap sites observed in the present 5′ RACE clones, it is likely that these short 1c transcripts are legitimate components of the BDNF mRNA pool in the cerebellum.

Nakayama et al. (1994) went a step further, proposing that in the rat brain, all exon 1c (exon II) variants are a composite of one, two or three short segments they termed exon 2a, 2b and 2c, with 2a being the most 5′ segment and 2c the most 3′ segment. Possible combinations of these segments included: 2a only; 2a + 2b; 2a + 2b + 2c and 2b + 2c. Since the exon 2c segment, which contains the present exon 1c, was never seen without the exon 2b segment, the splicing scheme for this exon remains controversial. Possible interpretations of the present data include: 1) the present 5′ RACE clones contain truncated versions of the exon 2 transcripts reported by Nakayama et al. (1994), perhaps due to an inability of the reverse transcriptase to consistently read upstream RNA sequence in this exon; 2) the present source of BDNF mRNA, the rat cerebellum, does not express exon 2a and expresses 2b sparingly.

The first interpretation is unlikely because Timmusk et al. (1993), like the Nakayama group, used hippocampal RNA in their studies and localized a number of putative translation start sites that would produce a transcript similar to that shown in Figure 3. Also, visual inspection of the sequence of the exon 1c region in the genomic clones of these investigators does not reveal particularly difficult reverse transcription templates, such as areas containing long stretches of CTP and/or GTP nucleotides. The second interpretation is more likely, given the

preponderance of data suggesting that the levels of alternate BDNF transcipts are differentially regulated in different cell types (Timmusk et al. 1993; Nakayama et al. 1994; Kokaia et al. 1994; this dissertation). Thus, exon 1c splice variants in the cerebellum appear to be truncated, as compared to analogous hippocampal transcripts. If the splice variants demonstrated by Nakayama et al. (1994) are common in other regions of the brain, and the present segment (exon 1c) is truly only found in approximately one half of these variants, then the present results may have underestimated exon 1c (exon II) transcript levels. However, the semi-quantitative PCR data suggested that levels of exon 1c mRNA in the hippocampus are greater than exon 1d levels in this brain region, and since both Nakayama et al. (1994) and Timmusk et al. (1993) reported that 1d levels were the most abundant under basal conditions, it seems unlikely that the present data underestimates exon 1c (exon II) transcript levels.

V-3.c. BDNF Exons 1d and 1e

The exon 1d- and exon 1e-containing BDNF transcripts obtained with 5′ RACE and shown in Figure 3, which were each observed in 3 or more of the 70 5′ RACE clones, exhibited appropriate lengths and sequences, as compared to the analogous exons (exons III and IV) described later (Timmusk et al. 1993; Nakayama et al. 1994). The 5′ end of the exon 1e sequence was 2 bp downstream of the exon IV transcription start site reported by Timmusk et al. (1993) and although the 5′ end of the exon 1d sequence was approximately 100 bp downstream of the most 5′ start site reported by these investigators for exon III, it was located in the midst of a cluster of strong alternative start sites. This suggests that the observed 5′ ends of the exon 1d and 1e-containing transcripts represent prominent translation start sites in the

cerebellum.

V-4 BDNF Alternate Exon mRNA Expression in the Substantia Nigra, Striatum, Hippocampus and Cerebellum

V-4.a. Overview

Determination of the nucleotide sequences of the five alternate BDNF first exons allowed the design and production of sequence-specific primers for use in semi-quantitative RT-PCR assays used to estimate the concentrations of each of these exons in the cumulative BDNF mRNA pool in different brain regions. In order to measure low abundance transcripts in these regions in individual rats, we developed a RT-PCR assay based on a method that has been successfully applied to quantitate the expression of the multidrug resistance gene *mdr-1* (Murphy et al. 1990). The present findings and evidence published by other investigators (Okazawa et al. 1992) indicate that BDNF mRNA can be consistently measured in regions of the basal ganglia and other regions where BDNF mRNA expression is low. Whereas others have compared BDNF alternate exon expression in hippocampal subregions (Timmusk et al. 1993; Nakayama et al. 1994; Kokaia et al. 1994; Metsis et al. 1993), in regions of the cerebral cortex (Timmusk et al. 1993; Kokaia et al. 1994; Metsis et al. 1993) and in peripheral tissues (Timmusk et al. 1993; Nakayama et al. 1994), the present work evaluated expression of these exons in the substantia nigra, striatum and cerebellum, as well as in the hippocampus.

V-4.b. Differential Expression of BDNF Alternate Transcripts

After determination of optimal input cDNA concentrations (Figures 7 and 8), BDNF 5' exon transcript levels were measured in substantia nigra, striatum, hippocampus and cerebellum relative to cyclophilin mRNA levels. For each alternate exon, the pattern of expression varied widely across brain regions. Although all BDNF transcripts were most abundant in the hippocampus, followed by cerebellum, substantia nigra and striatum, marked differences in the proportions of each were measured (Figure 9). It is evident that the prominent transcript in the substantia nigra contains exon 1c with approximately equal, but lower levels of expression of the other four alternate BDNF transcripts. Exon 1b transcripts were nearly as abundant in the substantia nigra as in the hippocampus, whereas the concentrations of the other BDNF transcripts were at least three-fold greater in the hippocampus than in the substantia nigra. These observations suggested that the promoter driving exon 1b is as active in the substantia nigra as it is in hippocampus and may therefore be a prominent promoter in this brain region. Since the dopaminergic neurons in the substantia nigra express BDNF (Seroogy et al. 1994), it is possible that BDNF mRNAs measured in RNA samples from this region were contained in the dopaminergic neurons, themselves. As another example of differential expression of BDNF alternate transcripts, levels of exons 1c, 1d, and 1e-containing transcripts in the cerebellum were approximately 2-fold greater than in the substantia nigra, whereas concentrations of exons 1a and 1b were lower in the cerebellum than in the nigra (Figure 9). The present work confirms the expression of BDNF in all four of the brain regions investigated and further suggests that mature *BDNF mRNAs made in the substantia nigra, hippocampus and cerebellum* are made up of all five alternate species discovered to date.

BDNF mRNA expression in the striatum also appears to result from differential regulation of alternate BDNF promoters. However, in the standard 30 cycle RT-PCR assays employed, even the most highly expressed exon, exon 1c, was barely detectable. In order to better estimate the extremely low levels of these exons in the striatum, additional PCR cycles were employed, revealing that the order of abundance of alternate BDNF transcripts in this region was 1c = 1e > 1a > 1b > 1d (Figure 10). It was also apparent that exon 1d-containing transcripts were much lower than previously thought and may actually be totally absent in this brain region. These observations corroborate previous reports that BDNF transcripts are sparce in the striatum (Maisonpierre et al. 1990; Okazawa et al. 1992) and suggest that exon 1c and 1e-containing transcripts account for the majority of this expression.

As predicted by the results of the 5′ RACE assays, the order of abundance of BDNF alternate transcripts in the cerebellum was 1c > 1e > 1d > 1a > 1b, which is distinctly different from that found in the striatum. Exons 1c, 1d and 1e were moderately to highly expressed in the cerebellum, whereas the expression of exons 1a and 1b were very low. In fact, these latter two transcripts were observed in only one and in none of the 5′ RACE clones derived from cerebellar mRNA, respectively, which is consistent with their relative scarcity in this brain region. The decision to search for alternate 5′ exons in a region other than the heavily investigated hippocampus apparently precluded discovery of the 1b exon, but allowed the discovery of the 1a exon.

V-4.c. Alternate BDNF Transcript Expression in the Hippocampus

Comparison of the expression of exons 1b-1e in hippocampus to values obtained by Timmusk and colleagues for exons I-IV, respectively (Timmusk et al.,

1993), revealed both similarities and differences. In our studies, exon 1b was weakly expressed in all regions investigated which is in concordance with the *in situ* hybridization studies conducted by Timmusk et al. (1993). Also, exon 1c (exon II) was highly expressed in hippocampus in both studies. However, we have measured relatively low concentrations of exon 1d in hippocampus (only exon 1b was lower), whereas the data obtained by Timmusk et al. (1993) suggests that constituitive expression of this exon is relatively high in this region. A possible explanation for this discrepancy is that, while exon 1d (exon III) is highly expressed in the dentate gyrus as well as the CA1 and CA3 regions, overall expression may be so low in other subregions of the hippocampus that the cumulative amount, which was measured in the present work, could be quite low. However, Nakayama et al. (1994) also reported that exon 1d is a prominent transcript in the hippocampus, based on ribonuclease protection assay (RPA) results obtained using total hippocampal RNA.

Conversely, Kokaia et al. (1994), while not specifically addressing constituitive alternate transcript levels, presented Northern blot evidence that the relative abundancies of the short (1.6 kb) splice variants of the 4 transcripts measured were: exon 1e > exon 1c > exon 1d > exon 1b. These investigators also showed *in situ* hybridization results wherein exon 1e-containing transcripts in hippocampal regions of control rats appeared to be found in greater abundance than BDNF transcripts containing exons 1b, 1c and 1d. Thus, the present results derived from RT-PCR assays of hippocampal total RNA are not easily aligned with the results of other investigators using other assays to measure alternate BDNF exon expression in this brain region. In the present study, exon 1e is found in intermediate concentrations in the hippocampus, whereas only low levels of this alternate exon were reported by Timmusk et al. (1993).

V-5 Dopaminergic Neuron Degeneration and BDNF mRNA Expression

Surprisingly, nigrostriatal lesions produced by unilateral injections of 6-OHDA had no statistically significant effects on BDNF mRNA expression in the striata of rats after 2 weeks or 3-6 month survival times. In both of these groups, BDNF/cyclophilin ratios were almost identical on the lesioned and unlesioned sides of the rat brains studied. However, in the 4 week survival group, these ratios were increased almost 2-fold on the lesioned side as compared to the unlesioned side (Figure 11). This effect was also observed for BDNF alternate exons 1a, 1c, 1d, and 1e (data not shown). However, since there was a decrease (about 2-fold) in cyclophilin mRNA expression in striata from the lesioned sides of the rats in the 4 week survival group that was not seen in the other two groups, it is likely that the observed increase in BDNF exon expression in this group is actually due to a lesion-related decrease in cyclophilin expression, rather than to a bona fide increase in BDNF transcript levels. Thus, although BDNF mRNA is manufactured in the striatum (Okazawa et al. 1992; this dissertation), and BDNF can protect nigro-striatal dopaminergic neurons from 6-OHDA induced degeneration in embryonic cultures of ventral mesencephalon (Spina et al. 1992; Fadda et al. 1993; Skaper et al. 1993), 6-OHDA induced lesions do not appear to result in long term compensatory increases in BDNF mRNA synthesis in this region *in vivo*.

Although 6-OHDA-induced dopaminergic cell death does not appear to stimulate long lasting BDNF mRNA synthesis (2 weeks or more) in the striatum, BDNF mRNA levels appear to be elevated at earlier time points in the substantia nigra (Numan and Seroogy, 1994). These investigators found that 8, 16 and 48 hours after injections of 6-OHDA into the medial forebrain bundle, increased BDNF mRNA hybridization densities in the substantia nigra and ventral tegmental area

occur. In contrast, no alterations in the hybridization densities for NT-3 or TH mRNAs were observed at these time points, suggesting that BDNF mRNA elevations were a specific result of the insult. It might be speculated that neuronal necrosis induced by the neurotoxin led to loss of the calcium potential, raising intracellular calcium levels and activating BDNF mRNA synthesis. BDNF produced in this manner is apparently not sufficient to reverse neurodegeneration, since the dopaminergic neurons do not survive. Interestingly, injection of 6-OHDA into the striatum also leads to degeneration of dopaminergic neurons, but requires a longer post-injection period to obtain lesions comparable to those obtained by injections into the medial forebrain bundle (Sauer et al. 1994). Perhaps BDNF synthesized in the nigra in response to degeneration induced by striatal 6-OHDA injections is involved in the slower progression of the lesion reported by these investigators.

V-6 Cellular Regulation of BDNF Gene Expression

V-6.a. Overview

Net expression of BDNF in the brain is dependent on the cumulative activities of its multiple alternate promoters and post-transcriptional processing (Timmusk et al. 1993; Nakayama et al. 1994). The alternate promoters are differentially responsive to physiological and biochemical challenges (reviewed in Lindvall et al. 1994) and, based on the present results, the C6 glioma cell line appears to be an appropriate cellular model for investigation of the roles of these promoters in the generation of increased BDNF mRNA levels resulting from such challenges. Studies conducted so far have demonstrated that several populations of neurons in

the brain respond to brain insults with increased BDNF mRNA levels (Isackson et al. 1991; Ernfors et al. 1991; Ballarin et al. 1991; Lindefors et al. 1991; Dugich-Djordjevic et al. 1992; Lindvall et al. 1992; Humpel et al. 1993; Rocamora et al. 1994) or electrolytic lesions (Gall, 1993; Kokaia et al. 1994) which has been attributed primarily to altered calcium homeostasis (Lindvall et al. 1994). Thus, neurons in the dentate gyrus of the hippocampus respond to kainic acid treatment with a large increase in a BDNF mRNA pool composed of exon 1b, 1c and 1d-containing transcripts (Timmusk et al. 1993; Metsis et al. 1993; Nakayama et al. 1994). Kainic acid-induced increases in BDNF transcripts in hippocampal neurons growing in culture were abolished by the calcium channel blocker, nifedipine, or the calmodulin antagonist, W7, strongly supporting the role of calcium in this process (Zafra et al. 1992).

In the present studies on the direct actions of calcium influx on alternate BDNF transcript expression, all of the transcripts measured were elevated in C6 cells 4 hours after treatment with the calcium ionophore, A23187 and the magnitude of this response was comparable to previously reported kainic acid-induced elevations of these transcripts in neurons (Timmusk et al. 1993; Metsis et al. 1993). Since A23187 produces calcium influx by simple diffusion through the plasma membrane, presumed calcium-gating mechanisms are essentially bypassed, allowing a gating mechanism-independent examination of the effects of calcium on BDNF (and GDNF) expression. The four hour incubation period was chosen to approximate the *in vivo* kainic acid injection paradigms reported in the literature (see Lindvall et al. 1994 for review).

V-6.b. BDNF Exon 1b and 1d Regulatory Mechanisms

Transcripts containing BDNF exons 1b and 1d in C6 cells were elevated by 15 to 30-fold and 8 to 12-fold, respectively, after a 4 hour treatment with A23187. This was comparable to the 30 to 50-fold and 12 to 16-fold elevations of the analogous transcripts observed in the hippocampus and regions of the cerebral cortex after kainic acid-induced seizures (Timmusk et al. 1993). Although the 1b and 1d promoters appear to be most responsive to calcium, the impact of increased 1b and 1d transcription rates on overall levels of BDNF coding transcripts in C6 cells is probably negligible due to their very low basal levels. Treatment of these cells with forskolin/IBMX or TPA had no effect on the expression of these transcripts, suggesting that PKA and PKC-mediated second messenger pathways are not significantly involved in activation of the 1b and 1d promoters in these cells.

V-6.c. BDNF Exon 1e Regulatory Mechanisms

Basal levels of BDNF coding transcripts in C6 cells are contributed mostly by exon 1e-containing transcripts. Assuming that the primers designed to measure these transcripts exhibit similar binding efficiencies, it is estimated that basal levels of exon 1e-containing transcripts are 2^5, or 32-fold, more abundant than their exon 1b or 1d-containing counterparts. The prevalence of exon 1e-containing transcripts in C6 glioma cells is congruent with previous reports that these transcripts are found primarily in non-neuronal cells (Timmusk et al. 1993; Nakayama et al. 1994).

Exon 1e-containing transcripts were elevated 8.3-fold and 3.7-fold after A23187 and forskolin/IBMX treatments, respectively, and thus appear to be responsible for

the bulk of the increase seen in the BDNF coding exon (6.4-fold and 2.5-fold, respectively). A23187-induced elevations in this transcript were unexpected since manipulations that increase intracellular calcium, such as kainic acid injection, KCl depolarization or seizure production in intact animals have had little or no effect on exon 1e-containing transcript levels in the hippocampus and other brain regions (reviewed in Lindvall et al. 1994). Perhaps smaller effects on exon 1e expression in non-neuronal cells could have been overlooked in these studies which generally focused on neuron-rich areas of the brain. Alternatively, 1e promoter induction by calcium might be a unique property of C6 glioma cells or of non-neuronal cells in general.

V-6.d. BDNF Exon 1a and 1c Regulatory Mechanisms

The expression levels of the other two known alternate BDNF exons, 1a and 1c, were very low in C6 cells, requiring 40 cyles of PCR to visualize a band. Since levels of background, nonspecific bands were unacceptable, these exons were excluded from data analysis. However, in one experiment, A23187 treatment produced approximately 5-fold elevations in the levels of each of these transcripts (data not shown). As suggested for exons 1b and 1d, 1a and 1c-containing transcripts probably make up a negligible proportion of the sum total of BDNF transcripts in C6 cells due to their almost nonexistant basal expression.

V-6.e. Possible Second Messenger Response Mechanisms Involved in the Regulation of BDNF mRNA Expression

Previous studies of mechanisms controlling BDNF expression have been

primarily concerned with the roles of excitatory amino acid pathways activated by various brain insults. Since calcium influx is an important step in excitation-induced BDNF expression (Zafra et al. 1992; Kokaia et al. 1994; Lindvall et al. 1994), and since BDNF protects cells from excitotoxic insults (Cheng and Mattson, 1994), calcium-dependent processess appear to be involved in both the regulation of BDNF gene expression and its neuroprotective actions. As discussed above, the calcium-induced increase in BDNF mRNA levels in C6 cells is ascribed primarily to increased exon 1e-containing transcripts. Inspection of the nucleotide sequence in the published 5′ flanking region of this exon, as well as the other BDNF alternate exons (Timmusk et al. 1993; Nakayama et al. 1994) failed to identify previously described calcium responsive consensus sequences, such as the calcium responsive element (CaRE, Sheng et al. 1988) or the serum responsive element (SRE, Sheng et al. 1988) found in the *c-fos* promoter (Ghosh et al. 1994). Thus, if a *cis*-acting element is directly involved in calcium-elicited increases in BDNF mRNA levels, it is either a novel one or is a previously reported element that has not yet been shown to confer calcium responsiveness.

Since activation of excitatory amino acid pathways can evoke c-fos expression prior to increased BDNF mRNA levels (Zafra et al. 1990), AP-1 could be formed in the appropriate time frame leading to transactivation of one or more of the alternate BDNF promoters. However, a functional AP-1 binding site in the rat BDNF gene remains to be identified and thus an indirect effect of calcium on the BDNF gene through AP-1 remains speculative. Moreover, treatment with TPA had no effect on 1e transcript levels, and since this compound activates AP-1 via activation of protein kinase C (Hunter and Karin, 1992), either sufficient AP-1 components (c-fos and c-jun proteins) are not expressed in C6 cells or the 1e promoter does not contain a functional TPA responsive element.

In addition to its response to elevated intracellular calcium, the 1e promoter is responsive to activation of the cAMP pathway in C6 cells, in spite of the lack of a cyclic AMP responsive element (CRE) in its 5′ flanking region. It is possible that adjacent BDNF promoters may share regulatory elements, as has recently been proposed (Timmusk et al. 1995). Thus, a CRE located just upstream of the exon 1d translation start site, while having no role in exon 1d mRNA expression, might be responsible for the observed cAMP-elicited increases in exon 1e expression.

V-6.f. Transcriptional Versus Post-Transcriptional Control of Exon 1e Expression

Actinomycin D significantly reduces A23187-induced BDNF mRNA expression, but not down to control levels (Figure 16), and since the dose of actinomycin D appeared to be sufficient to block transcription completely (Figure 16C and 16D), the remaining BDNF coding exon transcripts must be stabilized by a calcium-dependent process. The sequence of exon 1e is extremely GC-rich, with one section of this sequence (nucleotides 1798-1835, Timmusk et al. 1993), exceeding 90% GC content. Computer analysis of the propensity of exon 1e to form secondary structures, revealed several regions that could form stem-loop structures *in vivo* (data not shown) and potentially inhibit translation (Kozac, 1991). Generally, mRNA must be translated before it is degraded (Sachs, 1993), suggesting that inefficient translation of 1e exon-containing transcripts would impede their degradation, increasing their cytosolic longevity. This would account for the observed elevations of 1e exon-containing mRNA in the experiments employing actinomycin D and indicates that, as opposed to exons 1b and 1d, which are regulated exclusively at the level of transcription, steady state 1e exon levels are a product of both transcriptional and post-transcriptional processes.

V-6.g. Summary

The foregoing observations and results reported by other laboratories provide strong evidence that regulation of BDNF mRNA expression involves differential activation of alternate promoters (Timmusk et al. 1993; Metsis et al. 1993; Nakayama et al. 1994; Kokaia et al. 1994). In C6 cells, the roles of individual alternate promoters in subsequent production of BDNF mRNA is largely dependent on basal activity levels. Based on the results of semi-quantitative RT-PCR assays, basal levels of exon 1e-containing BDNF transcripts are substantially higher than all of the others combined. Theoretically, it would take a 32-fold elevation in exon 1b and/or exon 1d levels to match basal exon 1e transcript levels. Thus, the cumulative effects of exon 1e promoter activity and post-transcriptional processing events are critical in determining the amount of BDNF mRNA available for translation.

Since they were significantly reduced by treatment with actinomycin D, calcium-induced increases in BDNF alternate exon levels primarily reflect activation of alternate BDNF promoters (Figure 16). Therefore, in C6 cells, these promoters must contain calcium responsive elements which have not yet been characterized. Clearly, when pathophysiological conditions arise, with commensurate loss of calcium homeostasis (Auer and Siesjo, 1988; Cheng and Mattson, 1994; Lindvall et al. 1994), biosynthesis of BDNF is a high priority activity, since all five promoters are induced by increased intracellular calcium.

V-7 BDNF Exon 1e Promoter Analysis

The present results confirm the importance of exon 1e promoter-driven transcription in the production of BDNF mRNA in C6 cells and define some of the

functional regions involved. The region contained in pCAT1e-5'.2, spanning nucleotides 1626-1800 (Timmusk et al. 1993), is sufficient for basal transcription in C6 cells and confers approximately 2-fold greater transcription of the CAT reporter sequence than the control vector (pCAT Basic). In addition to CAAT and TATA boxes located at nucleotides 1732 and 1756, respectively, this region contains several previously described cis-acting elements, including two consensus Sp1 binding sites, a PEA3 site and other sites homologous to AP-1, AP-3 and AP-4 (Table 3). It is likely that one or more of these elements plays a role in basal 1e promoter activity. Alternatively, basal activity may be conferred by an as yet uncharacterized element or elements. Further promoter deletion studies will be required to localize the key element(s). Since transfection of pCAT1e-5'.2 produced greater increases in CAT enzyme than transfection of the next longest construct, pCAT1e-5'.4, it appears that at least one negative regulatory element exists between 658 and 454 bp upstream of the 3' end of exon 1e, corresponding to nucleotides 1421-1626 of the published sequence (Timmusk et al. 1993). Potential previously described elements situated in this region of the exon 1e promoter include: an Adh1-US2 element at nucleotide 1565, a Ma1T box at 1438 and several other elements homologous to AP-4, CTF/NF1, E2aE-CB and E4TF1 (Table 3). Although none of these elements are documented silencers, positive regulatory elements can function as silencers as well, depending on the context within which the operate. For example, Minowa et al. (1994) have shown that neuroblastoma cells (NB41A3) contain a protein or proteins that can down-regulate transcription of the rat D_2 dopamine receptor gene by binding to an Sp1 site, which is generally considered to be a positive regulatory element.

At this juncture, only the consensus glucocorticoid response element (GRE) located 420 bp upstream of the exon 1e cap site has been suggested as a mediator of exon 1e transcription (Funakoshi et al. 1993). Transient transfection of pCAT1e-5'.6,

Table 3. Previously described DNA elements located in the exon 1e promoter region are listed along with their consensus sequences, the observed exon 1e sequence and the location in the sequence published by Timmusk et al. (1993). pCAT1e-5'.2 contained an insert that spanned nucleotides 1626-1800 and pCAT1e-5'.4 contained an additional 205 nucleotides upstream of sequence in pCAT1e-5'.2 (nt 1421-1626). According to the transient transfection data (section IV-5.d) pCAT1e-5'.2 contains the exon 1e minimal promoter and pCAT1e-5'.4 contains a negative regulatory element.

	Adh1-US2	AP1	AP3	AP4a
Consensus	CCCCGG	TTAGTCA	GGGTGTGGAAAG	CAGCTGTGG
Observed	CCCCGG	TTGGTCA	GGGAGGGGATAG	CAGCAGAGG
Location	nt 1565	nt 1735	nt 1705	nt 1523

	AP4b	CTF/NF1a	CTF/NF1b	E2aE-CB
Consensus	CAGCAGAGG	GGCTTCTAGCCAA	GGCTTCTAGCCAA	TGGGAATT
Observed	GAGCTTTGG	TGCTTAACCCCAA	CGGCTTCTGTCCAT	GGGGAATT
Location	nt 1541	nt 1558	nt 1586	nt 1592

	E4TF1a	E4TF1b	MalT	PEA3
Consensus	GGAAGTG	GGAAGTG	GGAGGGA	AGGAAA
Observed	GGAATTG	GGAAATG	GGAGGGA	AGGAAA
Location	nt 1480	nt 1569	nt 1438	nt 1639

	Sp1a	Sp1b
Consensus	GGCGG	GGCGG
Observed	GGCGG	GGCGG
Location	nt 1674	nt 1716

which contains this sequence, did not produce measurable CAT expression in C6 cells, which can probably be attributed to the different cellular models employed or to the fact that activation of the glucocorticoid receptor was not attempted in the present work. In addition, the recently described estrogen response element (ERE) located in the area of the translation start codon at the 5′ end of the BDNF coding exon (Sohrabji et al. 1995) may also regulate expression of transcripts containing exon 1e.

Two studies of the transcriptional regulatory mechanisms involved in BDNF gene expression have been reported to date. Timmusk et al. (1993) developed CAT reporter vectors containing the putative promoter regions of BDNF exons I-IV, corresponding to the exon 1b-1e promoter regions in the present work. Transfection of these vectors into rat C6 glioma cells, the mouse cholinergic cell line SN6 or the mouse Sertoli cell line TM4 led to varying increases in CAT activity in transfected cells. These results indicated that promoter strengths in C6 cells were exon I (1b) = exon IV (1e) > III (1d) >> exon II (1c), whereas in SN6 cells this order was exon IV (1e) > exon III (1d) > exon I (1b) > exon II (1c). TM4 cells appear to be devoid BDNF gene regulatory machinery, since none of these constructs produced detectable CAT activity, and this is in line with the assertion by Timmusk and coworkers that these cells do not contain BDNF transcripts. These findings provide further support for cell type-specific regulation of BDNF mRNA expression via differential activation of alternate promoters.

The C6 cell transfection results concerning exon IV (1e) reported by these authors are in contrast to the present results which indicated that transfection of 5′-extended versions of the exon IV (1e) promoter does not produce significant concentrations of CAT enzyme. The extent to which this discrepancy may be attributed to measurement of CAT activity (Timmusk et al. 1993) versus measuring

levels of the CAT enzyme itself is unknown. Another discrepancy between the results of Timmusk et al. (1993) and the present work is the assertion by the former that the exon I (1b) promoter is as active in unstimulated C6 cells as is the exon IV (1e) promoter. This contradicts both the results of this dissertation and their own assertion that only exon III (1d) transcripts are expressed in these cells (Timmusk et al. 1993). From the perspective of the present dissertation, it is unlikely that the exon I (1b) promoter is as active as the exon IV (1e) promoter in C6 cells, since basal levels of exon 1b-containing transcripts are difficult to detect, even with RT-PCR, and production of these transcripts is regulated solely at the level of transcription (Figure 16C). Perhaps the harsh conditions necessary to transfer DNA constructs into cells, involving large concentrations of calcium, has a long lasting effect on the exon I (1b) promoter.

Recently, Timmusk et al. (1995) generated transgenic mice harboring BDNF alternate promoters and evaluated the reponse of these promoters to various insults, including administration of kainic acid, spreading depression via application of KCl to the surface of the cortex and sciatic nerve transection. In general, the results of these experiments mimicked those obtained in excitotoxin-treated rats, in that the most highly inducible promoters were the exon I (1b) and exon III (1d) promoters located in insult-sensitive regions of the hippocampus and the cortex. Thus, these promoters can function autonomously in a tissue-specific manner and most, if not all, of the DNA elements responsible for insult-elicited induction are located within a few kilobases of the transcription start sites of BDNF alternate exons. A particularly interesting finding was that transection of the sciatic nerve led to a 5-fold increase in transgenic exon IV (1e) induction in the distal segment, which was attributed to increased expression of this exon in Schwann cells encircling the nerve. This lends further support to the hypothesis that the exon IV

(1e) promoter is predominantly involved in regulation of BDNF mRNA expression in non-neuronal cells and indicates that activation of this promoter is important for the compensatory response to axotomy. These investigators also reported that expression of the exon IV (1e) transgene mRNA in the cortex was significantly increased after application of KCl or kainic acid, but was unaffected in the hippocampus after kainic acid treatment, which compares favorably with the results of *in vivo* rat studies conducted by these investigators (Timmusk et al. 1993). These observations also suggest that the mechanisms of action of these two insults are neuron-specific, in spite of the fact that both lead to neuronal depolarization and calcium influx (Lindvall et al. 1994).

To summarize, the present results indicate that only a short proximal segment of the BDNF exon 1e promoter region is required for expression of this exon in C6 glioma cells and suggest that an adjacent region contains a transcription silencer. Further experiments will be required to isolate both positive and negative regulatory elements in these regions as well as to identify putative CaREs.

V-8 Cellular Regulation of GDNF Gene Expression

V-8.a. Overview

In 1990, Niijima et al. (1990) reported that extracts from striata dissected out of 6-OHDA-lesioned rats prolonged the survival of fetal dopaminergic neurons to a greater extent than extracts from unlesioned controls. Moreover, striatal extracts from Parkinson's disease patients are also capable of extending the longevity of cultures of dopaminergic neurons, whereas cerebellar extracts from these patients or extracts from control striata were ineffective (Carvey et al. 1993). Since BDNF

expression in the adult striatum is minimal, another factor or factors was suspected and pursued here. In their investigations of the trophic actions of conditioned media from cultures of primary glial cells on fetal dopaminergic neurons, Lin et al. (1993) isolated GDNF, which was later found to be enriched in the adult rat (Schaar et al. 1993; Choi-Lundberg and Bohn, 1995) and human (Schaar et al. 1994) striatum. Although additional known or unknown neurotrophic factors specific for dopaminergic neurons may exist in the striatum, GDNF has impressive actions on these cells (Lindsay, 1995). In addition to the protective actions of GDNF administered prior to surgical or chemical ablation of neurons, this factor is currently the only one with demonstrated restorative functions in vivo (Hoffer et al. 1994; Tomac et al. 1995a; Gash et al. 1995). Although the trophic and neuroprotective effects of GDNF are well recognized, the signal transduction mechanisms regulating its endogenous expression have not been adequately addressed.

V-8.b. Possible Second Messenger Response Mechanisms Involved in the Regulation of GDNF mRNA Expression

In the present study, marked increases in GDNF mRNA concentrations were seen after A23187-induced calcium influx (Figure 17). These findings are comparable to those reported by other laboratories investigating the effects of seizure activity on expression of this transcript because a common result of seizure activity is loss of calcium homeostasis and calcium influx (Auer and Siesjo, 1988). Humpel and coworkers (Humpel et al. 1994) reported that kainic acid-induced seizures result in elevated GDNF mRNA levels in neurons of the dentate gyrus within 3 hours and Schmidt-Kastner et al. (1994) reported similar elevations in the striatum,

hippocampus and cortex following pilocarpine-induced seizures. Since these observations are comparable to the data obtained in the present work, the C6 glioma cell line appears to be a good model system to study the role of excitotoxic insults on GDNF expression.

In addition to the effects of increased intracellular calcium, the present study found that TPA treatment resulted in an approximately 3-fold increase in GDNF mRNA levels and that forskolin/IBMX treatment had no effect (Figure 17). Thus, in C6 cells, the GDNF gene is moderately responsive to PKC activation and unresponsive to increases in intracellular cAMP. Since PKC substrates include a number of transcription factors, such as c-fos and c-jun (see Hunter and Karin, 1992 for review), activation of this enzyme may have a role in the regulation of GDNF gene transcription. At the present time, the genomic sequence of the GDNF gene and associated 5'-flanking region has not been reported so consensus *cis*-acting elements involved in calcium and PKC regulated transcription (such as a cyclic AMP responsive element or a TPA responsive element) have not been identified.

V-8.c. Transcriptional Versus Post-Transcriptional Control of GDNF mRNA Expression

Experiments employing the RNA polymerase inhibitor actinomycin D suggested that GDNF transcripts in C6 cells are subject to a high turnover rate indicated by abolition of basal expression by the end of the four hour treatment period (Figure 18). Since the actinomycin D dose was sufficient to completely block RNA polymerase II-mediated transcription, as was suggested by the data concerning alternate BDNF exons 1b and 1d (Figure 16C and 16D), GDNF mRNA molecules remaining after A23187 + actinomycin D treatment are most likely due to calcium-

mediated stabilization of GDNF transcripts. As depicted in Figure 18, A23187-induced GDNF transcripts remained elevated by 2.4-fold in spite of total abolition of basal levels under the same conditions. Thus, post-transcriptional processing apparently plays a major role in the regulation of GDNF expression in C6 cells. The extent to which these data will generalize to CNS neurons or glia *in vivo* remains to be determined.

V-9 Conclusions

Over the past few years, it has become increasingly clear that growth factors play a major role in the development and maintenance of the nervous system. BDNF and GDNF have potent trophic and neuroprotective effects on a variety of neuronal populations, including those implicated in Alzheimer's disease and Parkinson's disease. Thus, evaluation of these factors for the treatment of neurodegenerative disorders is of high priority in the biomedical research community.

This dissertation investigated the intracellular mechanisms involved in the regulation of BDNF and GDNF mRNA expression, using both a rat model and a tissue culture model. The primary observations included: 1) the existence of a novel BDNF alternate exon (1a); 2) differential regulation of five alternate BDNF transcripts in different regions of the rat brain; 3) evidence that the presumed compensatory response to 6-OHDA lesions does not involve long term elevations in the expression of BDNF transcripts in the striatum; 4) evidence that calcium is the prominent second messenger involved in the activation of transcription of all of the BDNF and GDNF transcripts investigated, and; 5) evidence that the expression of both BDNF and GDNF mRNA is regulated primarily at the level of

transcription, but that BDNF trancripts originating from the exon 1e promoter , as well as GDNF transcripts, can also be modulated by post-transcriptional mechanisms.

This study and others have indicated that calcium-mediated signal transduction pathways are crucial in the regulation of BDNF and GDNF expression. Elucidation of calcium-dependent mechanisms controlling the transcription of these genes and post-transcriptional processing steps will facilitate our efforts to augment these factors for therapeutic purposes. Classical transmitter replacement stategies for neurodegenerative disorders, such as Parkinson's disease, have many shortcomings including adverse effects that become prominent in advanced stages (Mouradian et al. 1987; Mouradian and Chase, 1988). Thus, the necessity for developing novel therapeutic approaches that retard the relentless neuronal death in these disorders is inceasingly realized. Future studies will further clarify the mechanisms by which extracellular signals activate transcription of the BDNF and GDNF genes. If active promoters can be further induced or repressed promoters can be derepressed, the concentration of BDNF and/or GDNF in a given brain region can be increased, with possible therapeutic applications.

Appendix

The following pages provide a detailed description of the RT-PCR controls utilized in all semi-quantitative RT-PCR assays conducted in this dissertation. Since quantitative PCR is a very recent method employed to measure mRNA levels, and is considered controversial by some investigators, rigorous controls are necessary if artifactual data is to be avoided. Due to the length of the description, it was deemed appropriate for an appendix, while a more concise description appears in section III.

RT-PCR Controls

Reverse Transcription Controls

Due to the geometric amplification of target cDNAs obtained by PCR, the potential for amplification of non-specific templates, resulting in misleading or uninterpretable data, is great. Therefore, RT-PCR controls are a necessary part of any attempt to quantitate mRNA levels in tissue extracts using this procedure. Since the procedures developed during the course of this dissertation were not yet documented in the literature, a careful disscussion of them is warranted. To ensure that only the correct PCR product was obtained from RT-PCR asssays, both the RT and the PCR steps employed were optimized by the inclusion of the proper controls and by generation of standard curves. The RT procedure was identical in all experiments, regardless of the target RNA, and all of the RT primers utilized had relatively low T_ms between 50 and 54^{o}C, which allowed the reverse transcriptase to copy the RNA template, but precluded participation of these primers in subsequent PCR amplification cycles because the latter are carried out at temperatures of 55^{o}C or greater. Thus, the resulting PCR products were visualized in agarose gels as single bands, as opposed to two bands that would occur if the RT primer were able to anneal with the target cDNA. Another important consideration involving the RT reaction was that an aliquot of each sample RNA was processed in the same manner as the the actual sample, except that the reverse transcriptase was omitted. If non-specific DNA, such as genomic DNA or PCR products carried over from previous experiments was present, one or more PCR products would be observed, whereas no bands would be observed after RT-PCR conducted on a sample that was free of

contaminating DNA. All RNA samples were screened using this procedure and determined to be free of contamination prior to inclusion in BDNF, GDNF or cyclophilin RT-PCR assays.

Primer Set Design

As a further precaution enlisted to avoid nonspecific DNA contamination in RT-PCR assays, 5′ primers for BDNF alternate exons were always paired with the same 3′ primer (P8), which targeted a 27 bp sequence in the coding exon. Only RT-PCR products generated from mRNA templates would be of the predicted size; products generated from genomic DNA could not be obtained due the the extremely long intron(s) (>17 kb) separating exons 1a-1e from the coding exon (see Figures 3 and 4). Unfortunately, this procedure could not be applied to the measurement of BDNF coding exon, GDNF or cyclophilin transcripts, since the published sequences of these transcripts are not interrupted by introns. As mentioned previously, an attempt was made to match PCR primer pairs to one another to facilitate intercomparison of RT-PCR results. T_ms were always between 72 and 78^{O}C and PCR cycle numbers and PCR conditions were held constant whenever possible. Annealing temperatures were selected after extensive pilot work and construction of cDNA concentration-percent incorporation curves.

In the studies of BDNF alternate exon expression in the rat brain, the annealing temperature and cycle numbers were set to 55^{O}C and 30 cycles, respectively. Except in the case of the GDNF primer set, which required a higher (64^{O}C) annealing temperature, all of the PCR primer sets utilized in semi-quantitative RT-PCR assays performed on extracts of C6 cells were subjected to

annealing temperatures of 58.5°C (primers P5 and P6) or 60°C (all other BDNF and cyclophilin primers). Based on the aforementioned adjustments to RT-PCR assays, and assuming that these adjustments normalized primer binding efficiencies across primer sets, comparison of the expression of each transcript in a given tissue was possible.

Cyclophilin RT-PCR

For every sample RNA analyzed in BDNF or GDNF RT-PCR assays, a parallel aliquot of this RNA was subjected to cyclophilin RT-PCR analysis. Cyclophilin was selected because it is expressed constituitively by practically all cells and is widely used to normalize results of Northern blot experiments (Danielson et al. 1988). The expression of other "housekeeping" genes, such as glyceraldehyde phosphate dehydrogenase (GAPDH) and dihydrofolate reductase (DHFR) were also investigated (data not shown). The GAPDH primers assessed, which were made in our laboratory based on the published sequence (Ercolani et al. 1988), did not reliably amplify GAPDH cDNA made from rat total RNA. It is likely that these primers, which were designed to amplify human GAPDH sequences, did not show adequate homology to the respective rat sequences. The DHFR primers, which target the mouse sequence and were purchased from Stratagene, (LaJolla CA) also failed to produce the proper PCR product in pilot experiments. Theoretically, variations in the concentrations of cyclophilin mRNA in different samples indicates variations in total RNA added to RT-PCR assay tubes. Thus, expression of RT-PCR results as the ratio of BDNF exon product to cyclophilin product can be used to normalize these intersample variations in RNA concentrations.

Concentration-Percent Incorporation Curves

Another important step required to estimate relative concentrations of sample RNA using the present semi-quantitative RT-PCR procedure is the construction of input cDNA concentration-percent incorporation curves as described in section III-3. Briefly, concentration-percent incorporation curves for BDNF, GDNF and cyclophilin assays were generated by plotting concentrations of input cDNA (expressed as a percentage of a standard 20 µl RT reaction) against amounts of ^{32}P-labeled primer counts incorporated into resultant PCR products. This procedure was repeated for each cDNA sample type (rat brain or cultured cell RNA) for each transcript measured (BDNF exon, GDNF or cyclophilin) and the resulting curves were used to select optimal cDNA concentrations for subsequent RT-PCR analyses.

For example, in the data depicted in the cyclophilin concentration-percent incorporation curve (Figure 7), an input cDNA volume of 0.04 µl, which is 0.2% of a standard 20 µl RT reaction, led to incorporation of 0.2% of the radioactive PCR primer. Since this data point fell in the middle of the linear portion of this curve, 0.2% of a standard RT reaction was an appropriate amount of input cDNA to use in cyclophilin RT-PCR assays. It should be emphasized that the RT-PCR assay used in the present work is not a quantitative assay, but only semi-quantitative because no attempt was made to quantify the actual concentration of input RNA or cDNA (e.g. fmol/µl). A truly quantitative assay would require the generation of standard curves plotting previously determined input RNA concentrations against the

percent incorporation of labeled primer into gene-specific PCR products. Alternatively, competitive RT-PCR also employing known input RNA concentrations could be used (Siebert and Larrick, 1992). However, for the purposes of the present dissertation, which intended to compare gene-specific mRNA levels in different brain regions and treatment conditions, the selected semi-quantitative procedures were adequate. For example, if a 5 µl aliquot of C6 cell cDNA generated from a vehicle-treated dish leads to incorporation of 1% of labeled BDNF primer and an identical aliquot of cDNA generated from A23187-treated cells leads to incorporation of 5% of added BDNF primer, then 5-fold (500%) more BDNF mRNA was present in the A23187-treated cells. Curves for all three types of transcripts analyzed were generated from C6 cell RNA, while only BDNF and cyclophilin curves were required for analysis of rat brain samples.

In summary, determination of the nucleotide sequences of the five alternate BDNF first exons has allowed the design and production of sequence-specific primers for use in semi-quantitatiave RT-PCR assays used to estimate the concentrations of each of these exons in the cumulative BDNF mRNA pool in different brain regions. Similar oligonucleotide design steps were taken to set up BDNF, GDNF and cyclophilin coding exon assays. Implimentation of the controls discussed above is imperative so that artifactual results returned from assays employing PCR can be avoided. These include the use of RT primers with relatively low melting temperatures (T_ms), parallel RT-PCR reactions omitting the RT enzyme, the use of intron-spanning PCR primers sets whenever possible, selection of an appropriate housekeeping gene to use for normalization of input RNA concentrations and strict attention to the input cDNA concentrations used so that assay linearity can be maintained. Use of matched pairs of PCR primers is also required to allow comparison of results generated by different primer sets.

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